TAMPERE UNIVERSITY OF TECHNOLOGY

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# ADVANCED METHODS FOR CULTURING NEURONAL CELLS WITH MICROSTRUCTURES

Master of Science Thesis

Examiners:
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# ABSTRACT

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During the development of the neural tissue of the nervous system, supporting neuroglial cells and the extracellular matrix (ECM) guide the migration of the immature functional neurons, provide them with a scaffold to grow on and aid in the formation of synapses. ECM provides the neurons with various guidance cues that guide the migration of the neurons and the extension of the neurites. The micrometre and even nanometre guidance cues can also be incorporated into neuronal cultures *in vitro* to study the effect of the guidance cues and to develop small neuronal networks with a desired architecture.

Three-dimensional neurocage structures were fabricated from Ormocomp®, a polymer-ceramic hybrid material, by two-photon polymerisation for this study. In this study these structures were tested with neuronal cells differentiated from human embryonic stem cells (hESC). The neurocages were attached to microscope glass slide samples, each sample containing approximately ten neurocages. The neurocages were first coated with laminin, an ECM protein, to enable the adhesion of the cells to the glass surface. The cell suspension was then applied and the cells were cultured to observe their growth and to study the guidance effects of the neurocages. During the study eight individual experiments were carried out to optimise both the application methods for the laminin solution and the cell suspension and the growth of the cells.

This study utilised both simple manual methods and two different micromanipulator set-ups in the application of the laminin solution and the cell suspension. It was concluded that the best method to apply the laminin solution was the micromanipulator set-up SU2, which utilised automated micromanipulator placement and pressure regulation. The cell suspension, on the other hand, could be easily applied onto the samples by placing droplets of the solution onto the medium covering each sample. As the cell population in the culture was small, conditioned medium taken from another neuronal cell culture was tested to increase the viability of the cell cultures of this study. The conditioned medium had a clear positive effect and the use of conditioned medium in future studies is therefore recommended.

During the study it was found that the cells initially inside the neurocages did not attach to the glass bottom even when the insides of the neurocages were accurately coated with the laminin solution. In contrast, the cells outside the neurocages generally attached to the glass bottom well and had a tendency to migrate towards the neurocages and over the structure walls into them. Therefore, it was concluded that the material Ormocomp® was not harmful to neuronal cells and it even seemed to attract them. Furthermore, the cells that had migrated into the neurocages readily stayed inside them and extended neurites along the edges of the neurocages. This indicated that the neurite guidance properties of the structures were very promising.

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Hermoston hermokudoksen kehityksen aikana hermoston tukisolut ja kypsymättömiä hermosoluja ympäröivä soluväliaine ohjaavat hermosolujen vaellusta, muodostavat niille kasvualustan ja avustavat synapsien muodostamisessa. Soluväliaine tarjoaa hermosoluille erilaisia ohjausvihjeitä, jotka ohjaavat niiden vaellusta ja viejähaarakkeiden ojentumista hermosolujen solukalvosta. Näitä mikro- ja nanometrikokoisia ohjausvihjeitä voidaan myös yhdistää hermosoluviljelmiin, jolloin voidaan tutkia erilaisten ohjausvihjeiden vaikutusta hermosoluihin ja muodostaa pieniä, halutun muodon omaavia hermoverkkoja.

Tätä tutkimusta varten valmistettiin kaksoisfotonipolymeroinnilla kolmiulotteisia neurohäkkirakenteita Ormcomp®:sta, joka on polymeeristä ja keraamista yhdistetty hybridimateriaali. Rakenteita tutkittiin hermosoluviljelmissä, joiden solut oli eristetty ihmisalkion kantasoluista (human embryonic stem cells, hESC). Neurohäkit päällystettiin laminiinilla, joka mahdollisti hermosolujen kiinnittymisen lasille, jolla neurohäkit olivat. Tämän jälkeen neurohäkeille lisättiin elatusaine ja solususpensio ja soluja viljeltiin rakenteilla. Viljelyn aikana tarkasteltiin sekä hermosolujen kasvua että neurohäkkirakenteiden vaikutusta solujen vaellukseen ja viejähaarakkeiden kasvuun. Tutkimukseen kuului kahdeksan erillistä koetta, joilla optimoitiin sekä laminiiniliuoksen ja solususpension lisäystä että solujen kasvuolosuhteita.

Tutkimuksessa hyödynnettiin sekä yksinkertaisia manuaalisia menetelmiä että kahta mikromanipulaattorilaitteistoa laminiiniliuoksen ja solususpension lisäyksessä. Tutkimuksen aikana todettiin, että paras menetelmä laminiiniliuoksen lisäyksiin oli mikromanipulaattorilaitteisto SU2, johon kuului automatisoitu mikromanipulaattorin ohjaus ja paineensäätely. Solususpensio sen sijaan oli helppo lisätä asettamalla suspensiopisaroita näytettä peittävän elatusaineen pinnalle. Koska viljelmissä käytetyt solumäärät olivat pieniä, toisesta hermosoluviljelmästä otettua konditioitua elatusainetta testattiin tutkimuksen soluviljelmien elinkyvyn lisäämiseksi. Konditioidulla elatusaineella oli selkeä positiivinen vaikutus ja sen käyttö tulevissa tutkimuksissa on siksi suositeltavaa.

Tutkimuksen aikana selvisi, että alun perin neurohäkkien sisälle laskeutuvat hermosolut eivät kiinnittyneet lasille edes silloin, kun neurohäkkien sisäpinnat oli päällystetty laminiinilla. Neurohäkkien ulkopuolella olevat hermosolut sen sijaan kiinnittyivät lasille hyvin jopa ilman laminiinia ja näyttivät pääsääntöisesti vaeltavan kohti neurohäkkejä ja jopa kiipeävän reunojen yli niiden sisäpuolelle. Tästä voitiin päätellä, että Ormocomp®-materiaali ei ole haitallista hermosoluille, vaan näytti olevan jopa hermosoluja houkuttelevaa. Neurohäkkien sisälle vaeltaneet hermosolut yleensä pysyivät niiden sisällä ja ojensivat viejähaarakkeita neurohäkkien seinämiä seuraten. Tämän perusteella rakenteet olivat hyvin lupaava vaihtoehto viejähaarakkeiden kasvunohjaukseen.

# PREFACE

This Master of Science thesis was done at the Department of Biomedical Engineering at Tampere University of Technology. The thesis was a part of a project called StemFunc, which incorporated the Department of Biomedical Engineering and the Department of Automation Science and Engineering at Tampere University of Technology and the Institute of Biomedical Technology at Tampere University (former Institute of Regenerative Medicine).

I wish to express my gratitude to Professor Minna Kellomäki and Docent Susanna Narkilahti for examining my thesis and for letting me work with this fascinating subject. I also wish to thank my supervisors Sanna Turunen (MSc, Tech.), Elli Käpylä (MSc, Tech.) and Laura Ylä-Outinen (MSc, Tech.) for guiding me through the whole project and especially for helping me with the experimental study. I also want to thank the people at the Department of Automation Science and Engineering for letting me work with their micromanipulator set-up with such a short notice and especially Juha Hirvonen for guiding me at the department. I also thank all the personnel at Hermia and IBT and especially the fellow thesis workers, who shared all the highs and the lows of this project with me.

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Minna Vittaniemi

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# ABBREVIATIONS

| | |
|---|---|
| BDNF | Brain-derived neurotrophic factor |
| bFGF | Basic fibroblast growth factor |
| CNS | Central nervous system |
| DRG | Dorsal root ganglia |
| ECM | Extra-cellular matrix |
| ESC | Embryonic stem cells |
| Ex | Embryonic day x<br>(for example, E1 = embryonic day 1) |
| GFAP | Glial fibrillary acidic protein |
| GRGDS | Peptide with the amino acid sequence glycine-arginine-glycine-aspartate-serine |
| hESC | Human embryonic stem cells |
| HUCB-NSC | Neural stem cells from human umbilical cord blood |
| MAP-2 | Microtubule-associated protein 2 |
| MEA | Microelectrode array |
| PDL | Poly-(D-lysine) |
| PDMS | Poly(dimethylsiloxane) |
| PEG | Poly(ethylene glycol) |
| PEO | Poly(ethylene oxide) |
| PLGA | Poly(lactide-*co*-glycolide) |
| PLL | Poly(L-lysine) |
| PLLA | Poly(L-lactide) |
| PNS | Peripheral nervous system |
| PSPI | Photosensitive poly(imide) |
| PTFE | Poly(tetrafluoroethylene) |
| Px | Postnatal day x<br>(for example, P1 = postnatal day 1) |
| SEM | Scanning electron microscopy |
| UV | Ultraviolet |

# 1. INTRODUCTION

The knowledge about the development and regeneration of neuronal networks is vital understand and find possible treatments for neuronal injuries and diseases, such as Alzheimer's or Parkinson's disease [21]. However, the neuronal networks present *in vivo* are too complex to be studied as such. Therefore the development of neuronal networks *in vitro* has become an important tool in this field of study, as such neuronal networks can be used to mimic the behaviour of neurons *in vivo*. [48] A useful non-invasive tool in the study of neuronal networks *in vitro* is a microelectrode array (MEA), which can be used to monitor both the structural and the functional development of a neuronal network. [49] However, a single electrode on MEA is not capable of monitoring the development of a single neuron, partly because of the migration of the cells on the MEA platform. To overcome this drawback, the possibility to combine various neuronal growth guidance cues with MEA has been studied. [11]

This study is a part of the project StemFunc (Biomimetic active environment for differentiating and maturing functional neurons and cardiomyocytes from stem cells), which aims to assemble a biomimetic cell culture platform that combines biochemical factors, mechanical strain, electrical stimulation and electrical activity measurements, gas and medium change via microfluidics and pH, oxygen and temperature sensors. The electrical activity measurements and the electrical stimulation of the project are conducted on the MEA platform. This study was performed to enhance the control of the growth and the orientation of the neuronal networks growing on the MEA. The novel biomaterial structures tested in this study could in future be fabricated on to the MEA, where they would guide the migration and neurite extension of the cells. This would give valuable information about the neuronal activity of individual neuronal cells.

The microstructures used in this study are two-photon polymerised onto microscope glass slide samples, which are then coated with laminin solution. Subsequently, the neuronal cells differentiated from human embryonic stem cells (hESC) are plated atop the samples and cultured for approximately a week. During the cell culture the effect of the microstructures on the growth and orientation of the cells is followed and after the culturing the samples are further studied with immunocytochemical staining. If all the methods used in the study are successful, the sample number is increased and the sample size decreased to enable more efficient sample testing. However, the optimisation of the methods to apply the laminin solution and the cell suspension may take a long time, as a similar study with human cells has not been conducted before.

# 2. NEURAL TISSUE

## 2.1. Overview of the nervous system

The nervous system is composed of neural tissue, supporting blood vessels and connective tissues. It can be divided into two parts: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of the brain and the spinal cord. It is in charge of the integration, processing and coordination of all the sensory data and motor commands in the body, as well as the higher-order functions such as memory, learning and intelligence. All neural tissue outside the CNS belongs to the PNS. This tissue, in cooperation with blood vessels and connective tissues, forms the nerves of the body. The PNS is a mediator of information between the CNS and the peripheral tissues. It delivers sensory information from the periphery to the CNS and motor commands from the CNS to the periphery. [40]

## 2.2. Neural tissue anatomy

The neural tissue can be roughly divided into two cell types: functional cells, neuronal cells or neurons, and supporting cells, neuroglia. All the information of the nervous systems is delivered by neuronal cells, while neuroglia assure that neurons can function properly. Neuroglia separate neurons from the surrounding tissue, protect them and form a supportive framework for them. They are also partly responsible for the significant difference between the neural tissue in the CNS and PNS as both the population and variety of neuroglia in these systems vary greatly from each other. [40]

### 2.2.1. Neurons

Neurons have the most diverse morphologies of all animal cells. The appearance of neurons varies from simple cells with one extending cellular process to highly complex cells with a myriad of processes and thousands of synapses. Such diversity is possible because developing neurons are able to extend long and branching cellular processes from the cell body or the soma. These growing processes, neurites, are essential to neurons in their function of intercellular communication. In mature neurons these processes can be divided into two types: axons and dendrites. Dendrites transmit information from other cells towards the soma and axons transmit information away from the soma to other cells. [19] The structure of a representative neuron is illustrated in Figure 1.

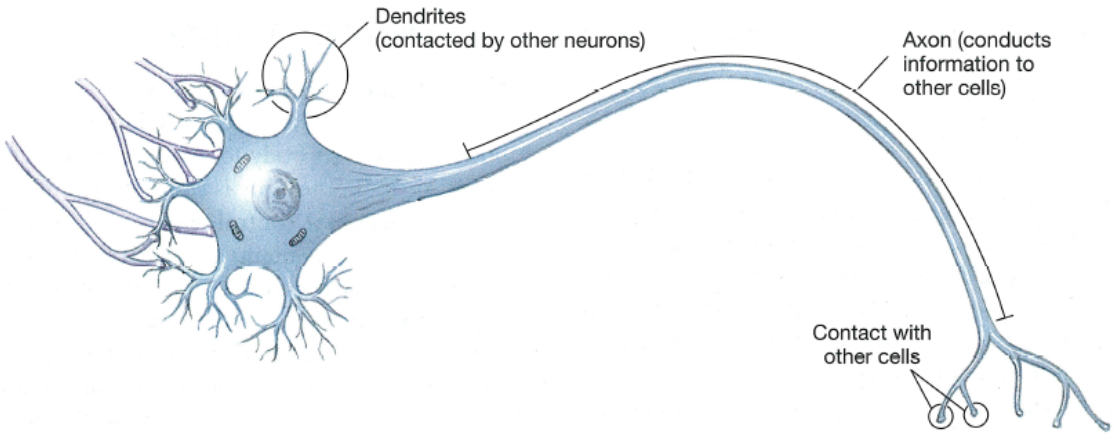



***Figure 1.*** *Structure of a neuron [modified from 40].*

As can be seen from Figure 1, dendrites are generally short and highly branched processes, whereas axons are long processes with only few branches. In the far end of the axon trunk are fine extensions, telodendria, which in turn end at the so-called synaptic terminals. Each synaptic terminal forms a synapse between the pre- and postsynaptic cell, where the information carried by the axon is passed on. [40] Neurons of the CNS usually only communicate with each other, whereas the neurons of the PNS also communicate with cell types other than neurons, such as muscle or secretory cells [19].

### 2.2.2. Neuroglia of the central nervous system

Neuroglia of the CNS consist of ependymal cells, astrocytes, oligodendrocytes and microglia. Astrocytes are the largest of neuroglial cells and form the majority in number. They have numerous functions in the CNS. [40] The fundamental role of astrocytes is to form a supporting matrix around neurons and provide them with nutrients and energy by ferrying oxygen and glucose from the blood. Additionally, astrocytes recycle the neurotransmitters secreted by neurons, thus contributing to the propagation of action potentials. [1] Astrocytes also contribute to the homeostasis of the brain by regulating the blood flow to the brain [21], by forming the blood-brain barrier and by stabilising the tissue after an injury. After an injury astrocytes move into the injury site and form scar tissue that prevents further damage. Unfortunately the scar tissue and the chemicals secreted by astrocytes at the damage site effectively prevent axons from regrowing across the damaged area. [40]

Oligodendrocytes are important for the structural organisation and functional performance of neurons. The cell membrane of the thin cytoplasmic processes of oligodendrocytes forms a very large sheet that gets wound around the axon, forming numerous layers of insulating wrapping called myelin. The myelin sheath insulates the axon from contact with the extracellular fluid and increases the travelling speed of an action potential along the axon. Each oligodendrocyte myelinates segments of several

axons and many oligodendrocytes are needed to form a complete myelin sheath along one axon. This cooperation of oligodendrocytes ties clusters of axons together and enables the extreme travelling speed of information in the CNS. [40]

### 2.2.3. Neuroglia of the peripheral nervous system

The neuroglia in the PNS consist of satellite cells and Schwann cells. The processes of these cells completely cover the somata and most of the axons of PNS neurons. Satellite cells surround the neuronal somata and cluster them into masses called ganglia. These cells also regulate the environment surrounding the neurons. [40]

Schwann cells are similar to oligodendrocytes in CNS, forming myelin sheaths around the neuronal axons. However, a single Schwann cell can only myelinate a segment of a single axon, whereas an oligodendrocyte can myelinate segments of several adjacent axons. A single Schwann cell can also enclose several unmyelinated axons. In addition to this function, Schwann cells are also important in the healing process of an injury in the PNS. [40] Unlike CNS axons, PNS axons can regenerate if the size on the lesion is no more than few millimetres and the tissues surrounding the nerve fibres are intact [53]. After an injury, the Schwann cells present at the injury site proliferate and form a cellular cord following the path of the original axon. The healing axon then grows along the cord and is myelinated by the Schwann cells. [40]

## 2.3. Development of neuronal cells and networks

The diverse shapes of all neurons are caused solely by the varying distribution of axons and dendrites in three-dimensional space. In vertebrates, neurons are generated during the development of the embryo and the process of neurogenesis is almost complete at the time of birth or hatching. Neurons originate from the outermost cell layer of the vertebrate embryo, the ectoderm, as unremarkable round cells. The extension of neurites, neuritogenesis, begins as a localised, highly active membrane protrusion at the surface of a neuron. The protrusion develops into a growth cone, a motile enlargement at the tip of the extending neurite. [19] Growth cones were discovered over a century ago by Ramón y Cajal, who described them as “battering rams that overcome obstacles along their journey to the targets of connectivity”. They are crucial to the developing nervous system as well as to the regeneration of damaged mature axons and dendrites. [50] A growth cone locates the cell that the neuron will form a synapse with and it builds the neurite behind it as it advances. When the other cell is located, the growth cone also forms the pre- or postsynaptic element, such as the synaptic terminal mentioned in Chapter 2.1.1. In summary, the growth cones establish the neuronal morphology and form the correct connections between neurons. [19]

In the last two decades significant progress has been made in understanding the mechanisms and molecules involved in the process of growth cone pathfinding [19], from the various guidance molecules and receptors to the cytoskeletal components mobilising the growth cone [50]. It is known that various interactions between the

developing neurons and their environment are involved in the differentiation and morphogenesis of neurons [31]. In the developing nervous tissue immature neurons are surrounded by glial cells that guide their migration to the correct location and provide them with a scaffold to grow on. This guidance is orchestrated primarily by special glial cells called radial glia. When a neuronal network is forming, astrocytes aid the neurons in synapse formation by secreting regulating factors and by direct contact with the neurons. Oligodendrocytes, Schwann cells and microglia also play a role in the guidance of synapse formation. [1]

In addition to the glial cells, neurons are surrounded by the extra-cellular matrix (ECM), which affects the migration and differentiation of immature neurons and guides the neurite extension of mature neurons [52] through various interactions with the cells. The adhesive interactions between cells and the ECM are best known. Adhesion is mediated by the binding of specific cell surface molecules, such as integrins, to the cell binding domains of ECM proteins. These bonds then stabilise the filopodia and lamellipodia of the cell and provide anchorage points to cytoskeletal filaments, which enables the movement of the cell or growth cone. However, the role of the ECM is not only to provide anchorage to the cells. The interactions between neurons and the ECM are complex and it is possible that ECM alone can regulate the movement and neuritogenesis of the cells. [31] In addition to the ECM molecules, the surface topography and three-dimensional architecture of the ECM are also important in guiding neurite growth and growth cone pathfinding, together with the soluble growth factors and endogenous electric fields present in the extracellular space [32, 49]. Despite the complexity of the molecules and other guidance cues present in the environment surrounding neurons, the growth cones are able to generate distinct responses to each of them [50].

# 3. NEURONAL NETWORKS *IN VITRO*

## 3.1. Cell source

Fragments of tissues were first studied outside the *in vivo* environment at the beginning of the twentieth century. Cells were first isolated from tissue samples only a few years later. Even today the common method of obtaining cells for culture is the isolation of tissue from an animal and the dissection or disaggregation of it to obtain the cells. A primary culture can be obtained by two different approaches. One approach is to let the cells migrate out from cultured tissue explants; the other is to mechanically or enzymatically disaggregate the tissue to produce a cell suspension. [14] Although mature neurons can be isolated from the brain, the cells isolated in most studies are neural stem cells. These cells can replicate into new stem cells and differentiate into all neuronal and glial cells even after long propagation periods [16].

Although the isolation of neural stem cells or mature neurons from the brain is a useful method to obtain material for neuronal cell cultures, it is impossible to dissect parts of living human brains. An alternative source of neuronal cells is to differentiate them from stem cells, such as embryonic stem cells (ESC). ESC are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos [47]. Pluripotency means that they can differentiate into all cell types of the body but cannot give rise to a new organism [16]. The developmental potential of ESC is unlimited. They can be propagated for years and yet they continue to replicate and remain capable of differentiating into any cell type of the body. [47]

### 3.1.1. Isolation of neural stem cells from animal tissue

The traditional method for obtaining a neural primary culture is the isolation and enzymatic disaggregation of brain tissue. Two excellent source tissues are the hippocampus and the subventricular zone. [16] According to the usual enzymatic disaggregation protocol, the tissue is digested by an enzyme, commonly papain, triturated and the obtained cells are isolated by density centrifugation [6]. Although some details vary, the protocol is essentially the same for both mature neurons and neural stem cells and for cells from various tissues or species [5, 6, 9, 36].

Although the enzymatic digestion protocol provides large numbers of cells, it has some drawbacks. The digestive abilities of the enzymes used must be carefully optimised as the enzymes giving the most complete disaggregation may damage the cells and the less harmful ones digest the tissue incompletely [14]. The protocol also demands high quantities of tissue and the microdissection of the target tissue alone

without the contaminating neighbour tissues has been proven difficult. To improve the anatomical resolution of neural stem cell isolation, Chipperfield and co-workers have developed a novel explant-based protocol, in which small tissue pieces are punched out of dissected tissue slices. They cultured the pieces and observed that cells of various types migrated out, including neural stem cells. The cell lines obtained from the progenitors could be expanded and maintained for months and finally differentiated into neurons and astroglial cells. [8]

### 3.1.2. Differentiation of neural progenitors from embryonic stem cells

The first ESC were isolated from mouse blastocysts in 1981 [18], after which the possibility to isolate primate and especially human ESCs has also been studied. The first human ESC (hESC) were isolated from human blastocysts and successfully cultured in 1998 by Thomson and co-workers [47]. At the time it was realised that hESC could be used to study the early development of humans [45] and later hESC have become a promising cell source for both basic research [20] and regenerative medicine [30]. Reubinoff and co-workers witnessed the spontaneous differentiation of hESC into neural progenitor cells in 2000 [45] and the first studies reporting induced differentiation of hESC were published a year later [54]. At present various protocols to differentiate hESC into both neural progenitor cells and specific neuronal and glial cells have been published [30].

hESC can be differentiated into neuronal cells by using a suspension culture, an adherent culture or a combination of these two [55]. A common suspension method is the culturing of embryoid bodies, floating aggregates derived from detached hESC colonies. When the embryoid bodies are plated and directed towards differentiation, they produce neural tube -like rosette structures containing neural stem cells, which can be further cultured to produce neuronal cells. [30, 54] Rosette structures can also be produced by an adherent culture method. In this method the hESC colonies are manually dissected and plated on for example Matrigel [2] or laminin [42] and cultured until the rosette structures are formed. The formed rosettes are then replated for further studies [2].

A simple suspension method is the direct neuronal differentiation of floating hESC aggregates without the embryonic body step. It has been used by various research groups [20, 30, 42] and is also used in the study of this thesis. In this method the hESC aggregates form round constant spheres called neurospheres. After a few weeks of suspension culture the neurospheres are enzymatically or manually disaggregated and the neural cells replated on laminin. This method is very simple to perform, cost-efficient and contains mostly controlled culturing steps, which makes it a promising candidate method to be used in large-scale hESC differentiation. [55]

## 3.2. Formation of neuronal networks

Although the isolation and differentiation of neuronal cells is a significant discovery, the individual cells themselves have little use. For their effective use in for example cell therapy they must develop and form functional neuronal networks. A neuronal network is a basic element of brain activity; a population of "synaptically interconnected neurons capable of generating electrophysiological activity that can spread spatially and temporally". [22] It has been shown that neurons isolated from mouse brain tissue retain their ability to form neuronal networks. When the isolated neurons are plated on a proper substrate, they readily grow and extend neurites to form a network containing large numbers of functional synapses. [49]

As a continuum to studies with isolated neurons, the functionality of single neuronal cells differentiated from both mouse ESC [3, 20, 22] and hESC [20] has been widely studied. Ban and co-workers were the first to show that neuronal cells differentiated from mouse ESC were able to form neuronal networks that behaved similarly to those derived from mouse primary cultures [3]. Shortly after, Illes and co-workers confirmed the potential of mouse ESC -derived neurons by reporting that the neuronal networks formed by these cells respond to pharmacological modifications in the same way as primary culture -derived neuronal networks [22]. In 2009, Heikkilä and co-workers reported that neurons differentiated from hESC also form functional networks *in vitro* [20] and their findings have also been confirmed in later studies [30].

## 3.3. Monitoring of neuronal networks

The accurate monitoring of the functionality of a neuronal network is at least as important as the actual functionality itself. Therefore, there is a need for a method enabling the accurate investigation of network dynamics. A microelectrode array (MEA) is a method for measuring the action potentials of neurons by extracellular electrodes. MEA is a completely non-invasive monitoring method where the electrodes are embedded into a growth plate and neurons can be monitored while culturing them on the plate. What makes MEA unique is its ability to measure the spatial and temporal distribution of electrical activity produced by the entire neuronal network. [3, 20, 22]

The culturing of neurons on MEA can give valuable information about the development of neuronal networks. As developing neurons extend their dendrites and axons and form synapses with other cells, a fully functional network is gradually formed. Both the structural (neurite outgrowth, synapse formation) and the functional (electrical activity) development of the network can be accurately monitored. [49]

## 3.4. Optimisation of neuronal network formation and monitoring

Although MEA is a highly useful method for monitoring neuronal network formation, the growth conditions are not ideal for neurons as they are cultured on a planar growth plate without the large population of glial cells present *in vivo*. The role of astrocytes in the formation of functional neuronal networks *in vitro* has been studied with neurons from various sources [44], even with those derived from hESC. Johnson and co-workers have studied the effect of exogenous astrocytes on the synapse formation of neurons and found that a monolayer of mouse cortical astrocytes significantly advanced the formation of synapses between the neurons [23]. However, Lappalainen and co-workers have reported development of synaptic activity in the same time scale as Johnson and co-workers with an almost homogeneous neuronal cell population containing only approximately 5 % astrocytes [30].

A major drawback of MEA is the lack of one-to-one correspondence between neurons and electrodes as a single electrode is not capable of monitoring the development of a single neuron. One reason for this is that neurons migrate during their first few weeks on MEA and the cells that are in contact with electrodes change. [11] First attempts to control the location of neurons on MEA have been made more than a decade ago. Maher and co-workers were one of the first to build a silicon micromachined device, a neurochip, which could monitor and stimulate neurons individually [38]. Unfortunately, the fabrication of their devices was very challenging and the popularity of them has been small. Nowadays there are numerous methods available for the controlled growth of developing neurons and many of the methods can be applied to MEAs, from simple chemical and topographical patterning (simulating the guidance molecules introduced in Chapter 2.3) to more complex three-dimensional structures [11].

# 4. NEURONAL GROWTH GUIDANCE *IN VITRO*

## 4.1. Neuronal guidance cues

As was mentioned in Chapter 2.3, the formation of neuronal networks is influenced by the numerous molecules and other guidance cues present in the local environment surrounding the developing neurons. As expected, the combined effect of the cues on neurons is very complex and not very easy to study *in vivo*. Hence, neuronal cell cultures have become a popular tool to study the *in vivo* environment by generating controlled microenvironments with specific features and isolated guidance cues. [32] Cell cultures are simple, fast and noninvasive when compared to *in vivo* experiments with live animals [35] and with them it is possible to study the specific interactions between various guidance cues and neurons [32].

Because traditional cell culture methods offer a homogeneous environment to all cells in the culture, the study of different factors affecting neuronal growth is laborious and time-consuming. Additionally, the cells in culture cannot be presented with multiple growth guidance cues in a competitive manner because of the homogeneous environment. To overcome these and other drawbacks of traditional cell cultures, microfabrication techniques, developed initially for the semiconductor industry, have gained interest in the *in vitro* study of neurons as well as various other cell types. With these techniques it is possible to design and control the culture environment accurately and study the effects of numerous variables simultaneously. Hence, microfabrication can be used to present neurons with specific patterns of guidance cues, which gives new information about the behaviour of neurons and enables accurate control of their growth. [35] The ability to apply directional control to neurites [32] and localise the cells to specific areas on the substrate [10] makes it possible to create small neuronal networks with a desired architecture. In these networks the growth patterns of the cells are defined, making it possible to trace individual cells and their synapses and study the propagation of the nerve signal in the network. [48]

### 4.1.1. Chemical guidance cues

Chemical modifications are made to the substrate surface by adding features that have a specific chemistry differing from the surrounding chemistry [48]. In the case of neuronal growth guidance, the chemical modifications are ECM proteins, soluble growth factors or other molecules that have a distinct effect on neurons. The molecules may be adhesion permissive or nonpermissive and biologically active or inactive. [10]

Biologically active and adhesive molecules include ECM proteins and cell-cell adhesion molecules that promote the adhesion of neuronal cells [10]. Examples of ECM proteins are laminin, collagen and fibronectin. Instead of whole proteins, it is also possible to use peptide sequences corresponding to the cell-binding sites of the proteins, although the peptides need to be quite long to provide the proper conformation of the cell binding site. Some of the widely studied include CDPGYIGSR, GQAASIKVAV, GRGDS and PHSRN. [52]

Biologically inactive molecules that promote cell adhesion can be used instead of ECM proteins to bind neurons to the substrate. Suitable candidates are polylysine and other positively charged polyaminoacids, aminofunctional groups attached to silanes or thiol-linking groups. Antiadhesive and biologically inactive molecules are generally used to confine cells into certain regions of the substrate. Generally these molecules are hydrophobic with the exception of poly(ethylene glycol) (PEG), which is highly hydrophilic and the most cell-repellent and protein-resistant molecule used in substrate coating *in vitro*. [10] However, coating with cell-repellent molecules is generally not necessary in neuronal cell cultures, because neurons are anchorage-dependant cells, meaning that they need an adhesive surface to be viable [12]. For this reason neurons that are cultured on a discontinuously coated substrate specifically adhere to the coated regions and direct their neurites to grow on them [32]. Hence, it is possible to generate various patterns of chemical guidance cues to limit the growth of neurons to certain regions of the substrate.

### 4.1.2. Topographical guidance cues

Topography refers to patterns of mechanical structures with a regular and specific size, shape and periodicity. It is the opposite of mechanical roughness, which is irregular and random regarding size, shape or periodicity. [48] Topography can be further divided to microtopography, which covers structures with dimensions between 1 µm and 1 mm, and nanotopography, which corresponds to dimensions less than 1 µm [43]. Similar to chemical guidance cues, topographical guidance cues can be used to specifically guide the growth and neuritogenesis of neurons *in vitro*. In contrast to chemical guidance, where neurons recognise the proteins adhered to the substrate, in topographical guidance they recognise elevations and drops on the substrate and can align themselves according to the topographical patterns. [52] Dimensions suitable for topographical guidance cues are discussed in Chapter 4.3.2.

## 4.2. Fabrication of neuronal guidance cues

The methods to microfabricate substrates suitable for cellular studies have been extensively reviewed in the literature [17, 28, 35, 43]. In this thesis the focus is on the most common patterning techniques that have been used to create patterns for neuronal cells. Chemical patterning sometimes needs to be combined with silanisation or other surface modification techniques to render the substrate surface adhesive for the

biomolecules. However, these methods are often complex and depend on the used molecules and substrate material. Hence, they are not discussed in this thesis.

### 4.2.1. Photolithography

Photolithography is the most widely used method of microfabrication. The photolithographic fabrication of a chemical or a topographical pattern begins with the fabrication of a so-called master. In this process the substrate is coated with a thin layer of a photoresist, a photosensitive polymer solution. Silicon or glass is usually used as the substrate. The pattern is created by exposing the photoresist to ultraviolet (UV) light through a photomask, which is a clear plate with the defined opaque pattern. The photoresist can be either a positive photoresist or a negative photoresist. UV light causes a positive photoresist polymer to break down and a negative photoresist polymer to crosslink. [35] After the UV light exposure the degraded positive photoresist or the non-crosslinked negative photoresist is dissolved with a suitable organic solvent, resulting in the defined polymer pattern on the substrate [25]. The resolution of the pattern can be as small as 1-2 μm and a resolution of tens of micrometres is easily accomplished [12].

After the fabrication of the master there are two possible ways to chemically pattern the substrate: lift-off and etching. In fact, the used method needs to be decided before the master fabrication because the application of the desired molecule, such as a protein or a peptide, occurs in a different stage depending on the method. In lift-off the substrate and the fabricated polymer master are coated with the protein, after which the photoresist master is dissolved in a proper organic solvent. In etching, on the other hand, parts of the substrate not covered by the master are removed before the photoresist is dissolved. Hence, in etching the substrate needs to be coated with the protein before the fabrication of the master to obtain the desired pattern. [46]

In addition to chemical patterning, photolithography can be used to create simple topographical features onto a substrate by a technique called greyscale topography. As the name implies, in this technique the clear mask is printed with a pattern incorporating multiple grey levels. A positive photoresist is used and as it is subjected to light through the mask, the various grey levels in the pattern produce features of different heights. [25]

### 4.2.2. Soft lithography

Like photolithography, soft lithography is a patterning method often used in biological applications. It consists of a group of techniques that are based on the use of an elastomeric material to act as a stamp, mould or a mask to create the patterns. The mould is created by making a replica of a preformed master that is usually fabricated photolithographically. The most common material of the mould is poly(dimethylsiloxane) (PDMS). Soft lithography includes techniques that are suitable for both chemical and topographical patterning. [48] Microcontact printing and microfluidic patterning are highly useful in chemical patterning, whereas

micromoulding and embossing are often used in the fabrication of topographical patterns.

Microcontact printing or microstamping is the most widely used technique among soft lithography. It uses the PDMS mould as a stamp to transfer "ink", such as a dried protein, onto a substrate in a specific pattern. The mould is first covered in a solution containing the molecules that then coat the mould surface. It is then pressed against the substrate and the molecules are transferred onto the substrate from the parts of the mould that are in contact with it. [48] The resolution of patterns fabricated with microcontact printing is around 1 μm [12].

Microfluidic patterning is a method similar to microcontact printing. In this technique, the PDMS mould is first placed on the substrate to form closed capillaries. The capillaries are filled with a solution containing the desired molecules that coat the walls of the capillaries. The mould is then removed, leaving the substrate patterned from areas that were not covered by the mould. [12] The resolution of microfluidic patterning is slightly poorer than that of microcontact printing as the dimensions of patterns created with microfluidic patterning range from 1 to 100 μm. Additionally, the patterns that can be fabricated are rather simple, such as parallel stripes. However, with this technique it is possible to pattern multiple different molecules in a single step, which makes it a useful patterning technique. [48]

Micromoulding is a common technique to fabricate topographical patterns onto substrates. In micromoulding, the void spaces of the mould are filled with a prepolymer in liquid state, the mould is placed on the substrate and the prepolymer is cured thermally or photochemically. After curing the PDMS mould can be peeled off, leaving the finished microstructures attached to the substrate. Micromoulding is a versatile and fast technique to create microstructures from various materials and it can be used with non-planar and curved substrates. However, the materials used need to be quite stiff, as soft materials, such as loosely crosslinked hydrogels, are damaged when the mould is peeled off. When fabricating microstructures from fragile materials, soft embossing can be used as the moulding technique. [48]

Embossing is a technique in which a pattern is fabricated onto the polymer substrate by pressing the patterned mould into the polymer and mechanically deforming the polymer to create the pattern. The material that is embossed needs to be either thermoplastic or curable by UV light or elevated temperature. [17] The mould can be rigid or, as in the case of soft embossing, elastomeric. In soft embossing the material is a partly crosslinked loose hydrogel that is cured after embossing with a PDMS mould. [48]

### 4.2.3. Photoimmobilisation

Photoimmobilisation is a method for attaching photoactive molecules onto a substrate with UV or laser light. Originally, the patterns have been created by covering the molecule layer with a photolithographic mask and exposing the molecules to light trough the mask. It is also possible to use a beam of laser light, which enables the

fabrication of more complex three-dimensional structures and heterogeneous patterns although this technique is a lot slower than the one using a photolithographic mask. [48]

### 4.2.4. Inkjet printing

Inkjet printing of biomolecule or cell solutions can be used to create chemical patterns onto various substrates. Commercial inkjet printers can be used to print biological solutions with minor modifications. The pattern is simply created by ejecting microscopic droplets of the solution from the printer head according to a defined pattern. The printer is completely computer-controlled, making this technique fast and flexible. The smallest possible drop size is approximately 100 μm and the resolution of this technique is generally in the range of 300-400 μm. [12, 48]

### 4.2.5. Laser ablation

Laser ablation is a patterning technique in which material is removed from the surface of a substrate with the use of a focused laser beam. In solid inorganic substrates, the photochemically excited gas created by the laser beam forms material radicals in the focal point of the beam. [17] Laser ablation can also be used with various biological polymers, where the laser beam breaks the covalent bonds in the polymer backbone and both photochemical and thermal degradation are involved. The resolution of laser ablation is about 0.1 μm and the depth of the etching can be effectively controlled. [48]

### 4.2.6. Two-photon polymerisation

Two-photon polymerisation can be used to fabricate random microstructures in three dimensions. It utilises the phenomenon of two-photon absorption, which initiates a polymerisation chain reaction in the monomer resin. Two-photon absorption is highly localised and polymerisation only occurs in the focal point of the laser beam. Furthermore, when the laser beam is moved inside the photosensitive monomer resin, the polymerisation takes place in the trace of the beam. [48] The resolution of features fabricated with two-photon polymerisation varies from study to study, as it depends on the characteristics of the material and laser system used. In general, resolutions as small as 100 nm can be achieved by using optimal conditions. [28]

The details of two-photon polymerisation have been thoroughly reviewed in the work of Käpylä and co-workers [27-29] and are hence not discussed in detail here. The details of the fabrication method used in this thesis can be found in [27, 29].

### 4.2.7. Electron beam lithography

Electron beam lithography is used to create patterns by locally exposing a photoresist covering a substrate to a beam of high-energy electrons. The electron beam can induce either solubilisation or polymerisation of the photoresist, leaving the desired pattern on the substrate. [43] Electron beam lithography can be used with various materials,

including inorganic materials and polymers. In polymers, the beam of electrons can induce polymerisation, crosslinking or local chain scission depending on the chemical composition of the polymer. [17]

The advantage of electron beam lithography compared to most of the techniques introduced above is that visible or UV light is not used in any stage of the technique. Photolithography and all other techniques utilising a photolithographic master have a definite lower limit of resolution: the wavelength of the light used. With electron beam lithography, it is possible to create single surface features with a resolution of a few nanometres and regular arrays of features with a resolution of tens of nanometres. However, the high resolution has its drawbacks as the fabrication of a pattern with electron beam lithography is both time-consuming and expensive compared to many other patterning techniques. [43]

### 4.2.8. Electrospinning

Electrospinning is a technique to create micro- and nanoscale fibres from organic and inorganic polymers, which can be used as scaffolds to guide the migration and growth of neuronal cells. In electrospinning, a droplet of a polymer melt or solution is suspended from a capillary and subjected to electric field. The electrostatic charge turns the droplet into a fine polymer jet. An electrically charged polymer fibre is formed when the solvent used evaporates and subsequently collected on a grounded surface. The fibres in the finished mesh are generally randomly oriented but a mesh of aligned fibres can be fabricated by collecting the forming fibres on a rotating plate. [43]

## 4.3. Applications of neuronal growth guidance

The first attempts to create a patterned network were made in the 1970s by Letourneau [37] and the possibility to force neurons to follow a defined pattern has been a dream of many scientists ever since. The various guidance cues and fabrication methods have given rise to a plethora of studies trying to make neuronal growth control easier to perform and more affordable. In this thesis a few studies are discussed in detail in an attempt to review the dimensions, patterns and materials feasible for the use in neuronal growth guidance. Some of the properties of the cell guidance systems discussed are presented in Table 1 (see Appendices) where the material, patterning technique and dimensions of various cell guidance systems are presented. Additionally, the cell type used is introduced and the effects of the guidance system on the adhesion and morphology of the cells are summarised. The various guidance systems are also discussed in more detail in the following chapters.

### 4.3.1. Chemical patterns and gradients

The molecules most commonly used in chemical growth guidance of neurons are the biologically unspecific polypeptide polylysine (both L and D enantiomers) and the ECM protein laminin. Polylysine is a cationic polypeptide that stimulates cell adhesion through nonspecific anionic-cationic interactions. Although the interaction between polylysine and neurons is not mediated by cell surface receptors [7], the two enantiomers PDL and PLL generally promote cell adhesion and neuritogenesis of neurons [15, 24, 34, 37]. Li and Folch have also studied the neuron-adhesive properties of Matrigel, a gel-forming basal membrane extract containing laminin and collagen I, on mouse embryonic cortical neurons. In their study, they concluded that Matrigel, too, can be used as a neuron-adhesive material, although its affinity for neurons is not as good as PDL's. [34]

Although neuronal growth guidance studies on human cells are rather scarce, Buzanska and co-workers have conducted an extensive study with HUCB-NSCs. They cultured these neural stem cells on both fibronectin and PLL and found out that although PLL did enable adhesion of cells on otherwise cell-repellent substrate, a majority of the cells stayed in the undifferentiated state. The ECM protein fibronectin, however, promoted neuronal differentiation and directionally guided the extension of neurites. [7] Therefore, it can be concluded that polylysine and Matrigel can be used to efficiently guide adhesion and neurite extension of non-human neurons, but the use of biologically active ECM proteins is necessary when culturing cells of human origin.

Because the adhesion of neurons to chemical guidance cues is very specific, the dimensions of guidance patterns can be greatly varied. Spots with dimensions of hundreds of micrometres can be used to pattern small subpopulations of cells [7, 37], whereas single cell somata can be located onto spots sized tens of micrometres [7, 15, 24]. Furthermore, even smaller spots or lines can be used to direct the growth of neurites, while the somata stay confined to the larger pattern regions. Jun and co-workers have studied neuronal growth guidance of rat embryonic hippocampal neurons with a PLL grid composed of 2 μm wide lines and 20 μm diameter nodes at line intersections. With a cell plating density of 100 cells/mm$^2$, nearly all cell somata were located at the nodes and the neurites extended atop the narrow lines. With higher plating densities, multiple cells and even small clusters of cells were observed on the nodes. These differences induced by different initial cell plating densities are illustrated in Figure 2. [24]

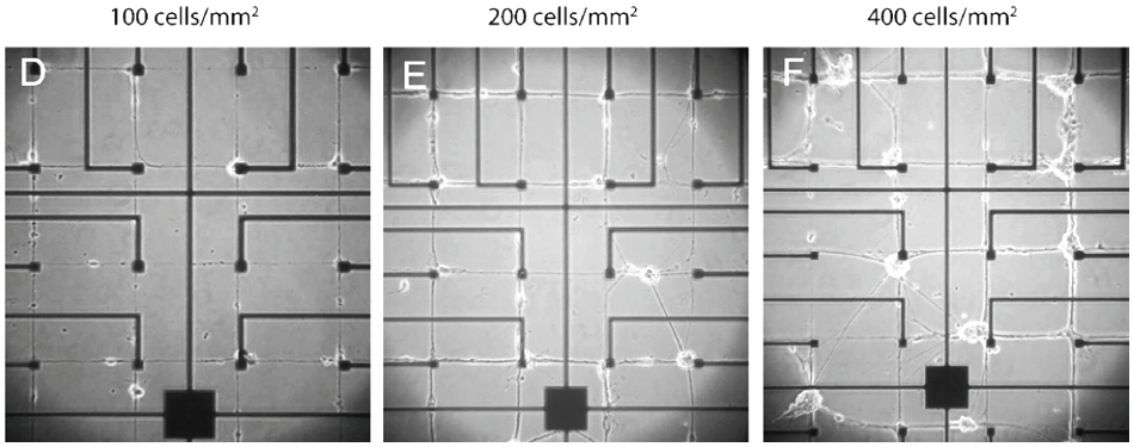



***Figure 2.*** *Rat embryonic hippocampal neurons on a PLL grid two weeks after plating [modified from 24].*

Fricke and co-workers, on the other hand, have studied the effect of PLL and laminin/PLL gradients in an attempt to specifically control the path and direction of extending axons. They found that a 200 µm gradient with increments of 0.3 µm and a 10 µm node in the middle resulted in longest neurites in the positive gradient direction with both PLL and laminin/PLL. For laminin/PLL wider gradients produced slightly longer neurites, whereas on PLL widening of the gradient had a negative effect on neurite length. The neurites were further immunostained for TAU-1 to identify axons. For laminin/PLL the most effective guidance was achieved with the narrowest gradients and the effect of the slope was not significant. On the contrary, on PLL gradients most axons were located on the wider gradients. In conclusion, almost 90 % of axons could be directed into the positive direction of the gradients by using these optimal gradient parameters. [15] However, it should be noted that the parameters favourable for neurite length were somewhat inhibitory to axons and parameters should be optimised to balance between these opposing effects.

When the effects of growth guidance cues are evaluated, it is important to reliably show that the cultured cells are indeed neurons. In the studies discussed here this was generally achieved by immunocytochemical staining of neuron-specific proteins in the cells. The proteins stained were α-tubulin [34], β-tubulin III [7] or microtubulin-associated protein 2 (MAP-2) [15, 24]. Macis and co-workers did not verify the identity of the cells by immunocytochemical staining but as the study was performed on a MEA [37], the electrical activity of the cells could be considered as a proof of them being neurons.

### 4.3.2. Topographical patterns

Topographical guidance patterns resemble the physical properties of the ECM. Therefore, it is natural that both the guidance cues and the material used have an effect on neuronal growth and migration. As can be seen from Table 1, both natural and

synthetic biocompatible materials have been used in topographical growth guidance of neurons. Polylactides have been an especially attractive material for neuronal growth guidance applications, because they are biodegradable and have been long used in tissue engineering applications [33, 51]. Furthermore, polylactides are quite easy to process with various methods [33] and they have permeability properties that enable good diffusion of nutrients in a cell culture [41]. However, the materials used in topographical growth guidance are rarely neuron-adhesive and are therefore generally coated with the adhesive proteins also used in chemical growth guidance (see Table 1 for details).

Both micro- and nanoscale dimensions have been utilised in topographical growth guidance of neurons, with somewhat inconsistent results. Li and Folch concluded in their study that neurons generally disregard grooves with heights 2.5 μm or 4.6 μm [34], a result that is contradictory with some of the studies discussed in this thesis and many others in the literature. Possible reasons for this discrepancy are the cell type, rat embryonic cortical neurons, and material, the elastomeric PDMS, used, both of which were different from all the other topographical patterning studies discussed here. Furthermore, the groove width used in the study (50-350 μm) was quite large when compared to the other studies and, more importantly, very large in comparison to the average size of a neuron. It is possible that growth cones cannot recognise vast, shallow grooves as guidance cues. The rather large width of even the narrowest grooves may also explain why the groove width did not influence axon turning in this study. [34]

As can be seen from Table 1, the studies performed on polylactides have produced similar results, although the pattern dimensions vary from study to study. Generally, the orientation of neurites can be affected with groove depths ranging from hundreds of nanometres [33] to several micrometres [34, 39, 41]. Neurons on topographically patterned polylactides extend longer neurites than cells grown on control substrates [33, 41] and at the same time the number of neurites per cell is sometimes diminished [39, 51]. However, the results presented in these studies cannot be fully confirmed, because the cells cultured were not generally identified as neurons by immunocytochemical staining. From the studies discussed here, only Li and Folch and Morelli and co-workers stained their samples with neuron-specific stains (α-tubulin and βIII-tubulin, respectively) [34, 41].

### 4.3.3. Structures confining cells

In addition to chemical and topographical guidance cues, developing neurons also encounter complex three-dimensional constraints *in vivo* [13]. As many other guidance cues, such constraints can also be fabricated for *in vitro* studies to elucidate the neuronal response to them. When the suitable dimensions are known, it is possible to fabricate structures that confine neurons to a certain location or force the cell migration and neuritogenesis to a certain direction on a substrate. Francisco and co-workers have studied the effect of physical constrains on the axon growth of chicken dorsal root ganglia neurons by culturing neurons in square and rectangular channels and on

corresponding two-dimensional surfaces. [13] The structures used in their study are shown in Figure 3.

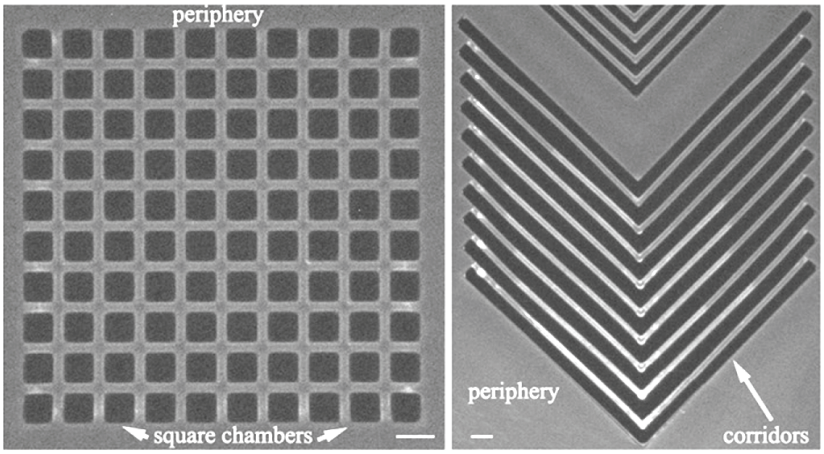



***Figure 3.*** *Three-dimensional chambers and corridors confining neurons [modified from 13].*

In the study only 40 % of neurons extended axons inside a square chamber sized 40 x 40 µm when compared to the axon extension of neurons growing outside the chambers. Furthermore, the percentage steadily increased to almost 80 % when the chamber size was increased from 40 x 40 µm to 70 x 70 µm. In comparison, when the neurons were cultured in long corridors with widths ranging from 20 to 50 µm, no significant difference to the control cells was observed. [13] From these results, it is obvious that the axon extension of neurons is somewhat hindered when the neurons are confined from all directions. The effect of confinement on axon length was studied by culturing cells in long rectangular corridors that were divided into square chambers and connected to each other by narrow "doors". It was concluded that the axon lengths of neurons cultured in these structures were approximately 30 % shorter than the axon lengths of neurons growing in rectangular structures without "doors". In conclusion, confinement of neurons can be used to control both the percentage of neurons extending axons and the length of the axons formed. [13]

### 4.3.4. Neuronal growth guidance on MEA platforms

Neurons can be localised atop MEA electrodes as single neurons or neuron subpopulations and with both chemical and physical confinement strategies. Macis and co-workers have conducted a study in which patterns of PLL or laminin/PLL were deposited on the electrodes with a piezoelectric droplet generator. Drop volumes ranging from 100 to 300 pl resulted in nodes with a mean diameter of 148 µm. They noticed that on MEA, PLL alone did not induce sufficient rat embryonic cortical neuron adhesion for the formation of interconnected neuronal sub-populations. For this reason, first laminin and then PLL were deposited on the same spot, after which neurons

generally adhered to the patterned regions and formed a functional network. However, some peripheral sub-populations were also formed and the node dimensions were less defined on MEA than on a glass controls because of the double drop deposition. A neuronal network after 20 days in culture on a MEA is shown in Figure 4. [37]

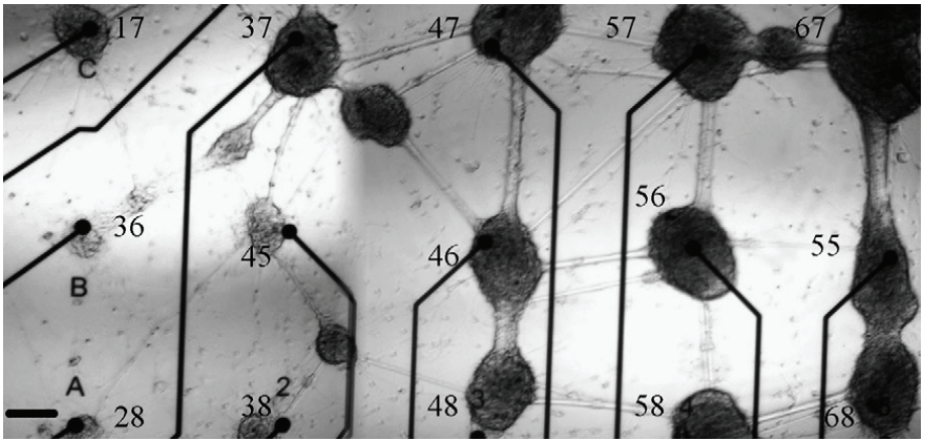



***Figure 4.*** *Neurons on a micropatterned MEA after 20 days [37].*

The functionality of the network was assessed by recording the spontaneous and evoked activity of the network at various time points (days 19, 25, 27 and 39). It was observed that recorded signals corresponded well to electrodes completely or partially covered by neurons. In conclusion, it is possible to use the microdrop delivery system to control the network architecture and subsequently the dynamics of the network. [37]

Jun and co-workers have also utilised a microcontact printed PLL pattern to control the neuronal adhesion and axon extension on a MEA. They coated the electrodes with 20-µm-diameter nodes of PLL and interconnected the nodes with a 200 x 200 µm grid of 2 µm wide lines. Their goal was to pattern one cell soma/electrode, which was achieved with an initial cell plating density of 100 cells/mm$^2$. However, this plating density resulted in few spontaneous signals from the neurons during the culture. The higher cell plating densities resulted in the formation of functional neuronal networks, but even with the highest cell plating density (400 cells/mm$^2$), less than half of the total electrodes were observed to be active. This could be due to a lack of sufficient synaptic input and trophic interactions between the cells. In future, the problem could be overcome by using electrical stimulation during network formation or suitable trophic factors or feeder cell layers to increase the formation of functional synapses. [24]

Berdondini and co-workers have designed a physical confinement structure to cluster a neuronal network on a MEA into interconnected subpopulations. The material used was the photoresist EPON SU-8, which is biocompatible and very easy to process with photolithography. The design of the clustering structure and a MEA chip with the structure are illustrated in Figure 5. [4]

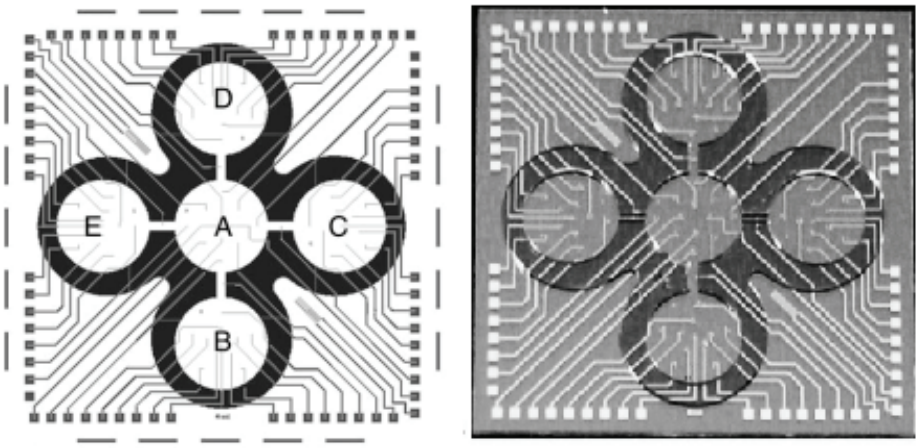



***Figure 5.*** *A design of a clustering structure (left) and the actual structure on a MEA chip (right) [modified from 4].*

The MEAs were coated with PLL and laminin prior to plating rat embryonic cortical neurons onto them. The neurons distributed evenly into the structures and remained active and healthy for up to 45-60 days. The mean levels of activity were very similar between the clustered MEA and a control MEA, but the bursting activity patterns between and within the clusters were found to be different as synchronous bursts in the clusters and asynchronous bursts between clusters were observed. This proved that the clustering structure could be used to confine the spontaneous activity of neurons into clusters. The evoked activity of the clustered network was also different from a control one, where a stimulus evokes a very similar response through the whole MEA. In the clustered MEA, however, the evoked activity was highly localised to the stimulated sub-population of neurons, but at the same time the activity was observed to spread to the connected subpopulations. In conclusion, the clustering structure could be used to successfully organise a neuronal network into interconnected subpopulations. However, the subpopulations in the study were very large as the cells were plated with an initial plating density of 1200 cells/mm$^2$. Therefore, the network could not be observed at a single-cell level. [4]

Erickson and co-workers have developed a neurocage structure similar to that developed by Maher and co-workers (discussed in Chapter 3.4) to confine single neurons atop MEA electrodes. Neurocage structures were fabricated from parylene by photolithography and placed around 16 electrodes on a MEA as shown in Figure 6. [11]

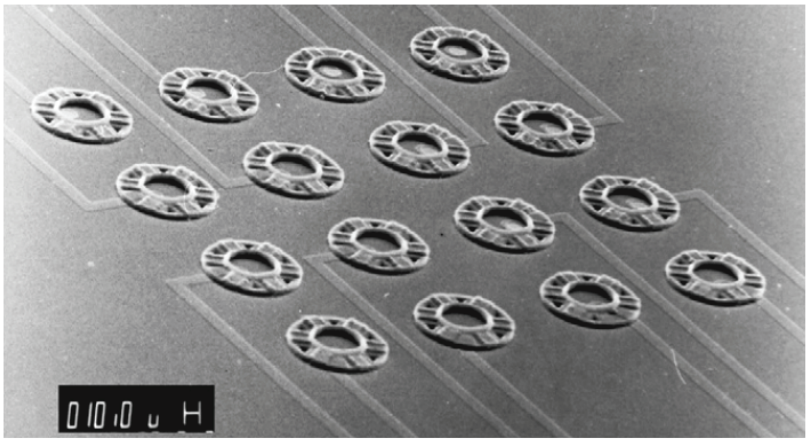


***Figure 6.*** *Parylene neurocages atop MEA electrodes [11].*

The neurocages were 40 µm in diameter and 9 µm high to fit the electrode and a neuron soma inside. Neurites were allowed to extend from the neurocages through 10 µm wide and 1 µm high tunnels. In the study, 11 out of 16 initial neurons were growing after ten days in culture and extended their neurites to form synapses with other neurons. A mass culture of 30 000 cells was used to condition the medium during the study to enable the survival of the minuscule population of neurons inside neurocages. The connectivity and evoked activity of the network were analysed and 29 out of 41 cultures developed observable connectivity. In conclusion, neurocages are a promising method to study neuronal network formation and function at a single-cell level but the formation of a functional network is not yet very reliable even with the highest of expertise. [11]

# 5. EXPERIMENTS

Neurocage fabrication steps were performed at VTT Tampere or at the Department of Biomedical Engineering at Tampere University of Technology. The application of laminin was performed either at the Institute of Biomedical Technology at Tampere University (IBT, formerly Regea, Institute of Regenerative Medicine) with the micromanipulator set-up 1 (SU1) or at the Department of Automation Science and Engineering at Tampere University of Technology with the micromanipulator set-up 2 (SU2). All the cell culturing was performed at IBT.

## 5.1. Fabrication of neurocages

The neurocages were fabricated for this study with essentially the same process as that described in [27, 29]. The material used was a hybrid polymer-ceramic material by the trade name of Ormocomp® (Micro Resist Technology, Berlin, Germany) with Irgacure® 127 (Ciba Specialty Chemicals, Basel, Switzerland) as an additional photoinitiator. Shortly, a 3D model of the neurocage was drawn and sliced to contours with Rhinoceros® CAD program (Robert McNeel & Associates, Seattle, USA) and the contour data was transferred to the LaserControlSystem software (VTT, Tampere, Finland). The Ormocomp® sample was then prepared by simple drop casting onto a microscope slide with five circular wells surrounded by a coating of poly(tetrafluoroethylene) (Electron Microscopy Sciences, Hatfield, USA) and polymerised as described previously [27]. As in the previous study, only the middle well of each microscope slide was used for polymerisation. After the fabrication of the neurocage structures, each sample was disinfected by soaking in 3 ml of 70 % (v/v) ethanol on a sterile 35 mm Falcon® EasyGrip™ Petri dish (Becton Dickinson Labware, Franklin Lakes, USA) for 15 minutes. The microscope slide was left to dry completely in a laminar flow hood and afterwards, each disinfected sample was moved to a new sterile 35 mm Petri dish.

In this study, two different neurocage designs, shown in Figure 7, were tested. The tunnel length in all of the neurocages was 40 µm, tunnel inner width 5 µm and node inner diameter 40 µm. Design A contained three collinear nodes with two tunnels connecting the nodes to each other. Design B contained four nodes in a square with a total of eight tunnels connecting the nodes to each other, four in a 90° angle and four in a 45° angle with respect to the centre of the node. For scanning electron microscopy (SEM) imaging, the neurocages were sputter coated with gold for 180 s corresponding to a thickness of approximately 113 nm with an Edwards S150 sputter coater (Edwards,

Sussex, UK) and imaged with a Philips XL-30 scanning electron microscope (Philips Electron Optics, Eindhoven, Holland). SEM images of the two neurocage designs are depicted in Figure 7.

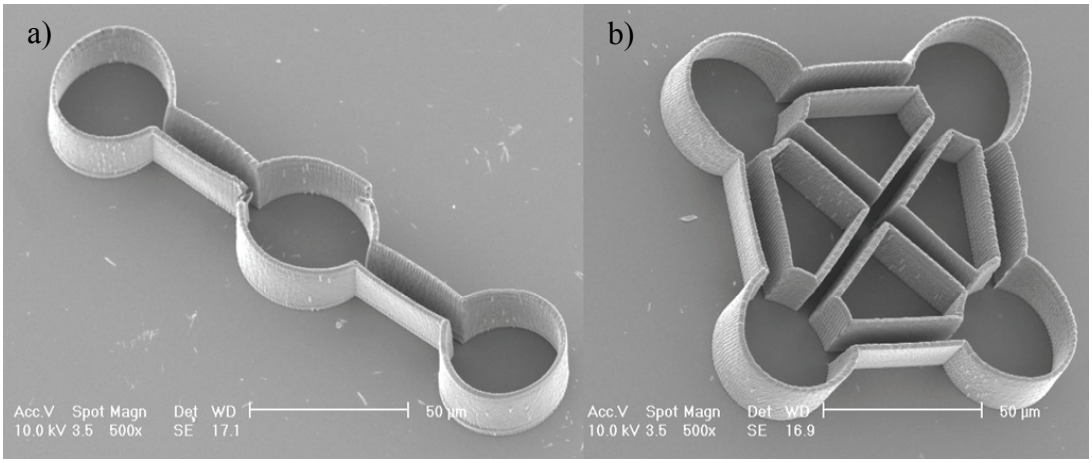
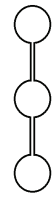
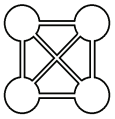



***Figure 7.*** *a) A SEM image of the neurocage design A. Tunnel l = 40 µm, tunnel inner w = 5 µm, node inner Ø = 40 µm. b) A SEM image of the neurocage design B. Horizontal and vertical tunnel l = 40 µm, tunnel inner w = 5 µm, node inner Ø = 40 µm.*

Each microscope slide sample contained 4-12 neurocages. The samples were labelled with two letters followed by consecutive numbering. The first letter described the material and the second letter the neurocage design used. The principles of labelling the samples are explained in Table 1.

***Table 2.*** *Labelling the samples.*

| **Material** | **Label** |
|---|---|
| Ormocomp® | O |
| **Design** | **Label** |
|  | A |
|  | B |

It was noted that the use of isopropanol as a solvent to remove the unpolymerised Ormocomp® (see [29] for details) often lead to the collapse of the tunnel walls due to the high surface tension of the alcohol. Although this phenomenon did not block the tunnels completely, hexamethyldisilazane (Sigma-Aldrich, Saint Louis, USA) was tested as an alternative solvent for samples OA2, OA4 and OA5. It was found that hexamethyldisilazane resulted in perfectly shaped tunnels and was used as the only solvent thereafter.

In addition to the fabrication phases already mentioned, there were a few optional phases that were suspected to affect cell viability and the durability of the neurocages. To assess these effects, four optional fabrication phases were tested. The optional phases were

1) soaking the samples overnight in sterile Dulbecco's phosphate buffered saline (DPBS, Lonza Group Ltd, Basel, Switzerland) after disinfection
2) coating the microscope glasses with 3-(trimethoxysilyl)propyl methacrylate (Sigma-Aldrich, Saint Louis, USA) before neurocage fabrication
3) baking the samples at 80°C on a hotplate for 3-5 min and subsequent treatment with UV light for 30 s after the neurocage fabrication
4) combination of phases 1 and 3.

## 5.2. Preparation of the neuronal cell suspension

Neuronal cells were differentiated from human embryonic stem cells (hESC) as described in [30]. The hESC cell lines used in this study were 08/023 and 06/040, both derived at IBT. IBT has ethical approvals to derivate, culture, and differentiate the human embryonic stem cells (Skottman, R05116), and the permission to human stem cell research from Valvira (1426/32/300/05). hESC colonies were mechanically dissected into small clusters that would form the neurospheres. The clusters were transferred into 6-well ultra low attachment plates (Nunc, Thermo Fisher Scientific, Rochester, USA) and cultured as flowing aggregates. The medium 0NDM [1:1 mixture of (1:1 mixture of Dulbecco's Modified Eagle Medium and Nutrient Mixture F12) and (Neurobasal Medium) supplemented with 2 mM GlutaMax, 20 µl/ml B27, 10 µl/ml N2 (all from Invitrogen, Carlsbad, USA) and 25 µg/ml penicillin/streptomycin (Lonza Group Ltd, Basel, Switzerland)] supplemented with 20 ng/ml basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, USA) was used as the neural differentiation medium. Medium was changed three times a week and the aggregates were mechanically passaged once a week. The aggregates were cultured for a total of 7-9 weeks, after which the differentiated neurospheres were used in the study. It had been found in previous studies with these cell lines that the proportion of astrocytes in the neurospheres began to increase after nine weeks of differentiation. Therefore the time frame used in the differentiation was rather precise.

Neurospheres were collected from one to four wells of the 6-well ultra low attachment plates depending on the amount of cells needed. Neurospheres in one well corresponded to approximately 500 000 neuronal cells in single cell suspension. The neurospheres were collected into a 1.5 ml Eppendorf tube (Eppendorf, Hamburg, Germany) and the medium was removed. The solution used in disaggregation was either TrypLE™ Select (Invitrogen, Eugene, USA) or trypsin (Trypsin-Versene 10x stock solution, 5 mg/ml trypsin, Lonza Group Ltd, Basel, Switzerland) diluted in DPBS to a 1x working solution. For TrypLE disaggregation, the neurospheres were suspended in 100 µl of TrypLE™ Select and the suspension was incubated for 15 minutes during which it was aspirated twice. After incubation, the suspension was aspirated with 1 ml of the medium 0NDM or 5+NDM [0NDM with 4 ng/ml bFGF and 5 ng/ml brain derived neurotrophic factor (BDNF, Invitrogen, Carlsbad, USA)]. For trypsin disaggregation, the neurospheres were suspended in 100 µl of 10 µg/ml trypsin solution. The suspension was incubated for 5 minutes, after which it was aspirated with 1 ml of 5 % (v/v) human serum (PAA Laboratories GmbH, Pasching, Austria) in DPBS. The suspension was centrifuged (200 g, 5 minutes) with an Eppendorf 5415D microcentrifuge (Eppendorf, Hamburg, Germany), the supernatant was removed and the cells were suspended in 1 ml of medium 5+NDM. After the preparation, the cell suspension was stored in the incubator and used during within four hours after preparation.

## 5.3. Experiment 1

At the time of the first experiment it was not certain whether any kind of solution could be inserted into the neurocages. Therefore, the concentration of the laminin solution and the amount of cells in suspension were chosen rather arbitrarily. Furthermore, very simple and effortless methods for the application of the laminin solution and the cell suspension were tested.

### 5.3.1. Materials and methods

Experiment 1 was performed in a Kojair BioWizard laminar hood (Kojair, Vilppula, Finland). A total of five neurocage samples of the design A were used. Each of the samples contained approximately ten neurocages. Two methods of cell suspension application were tested with samples OA1 and OA2. Subsequently, the suspension was added onto samples OA3, OA4 and OA5 with the method that produced better results with samples OA1 and OA2.

A solution of 20 µg/ml of mouse laminin (Sigma-Aldrich, Saint Louis, USA) in DPBS was placed on the samples with a simple droplet method. First, the neurocages on each sample were located by viewing the sample under a Nikon SMZ200 microscope (Nikon Corporation, Tokyo, Japan). A 20 µl droplet of the laminin solution was carefully placed on the glass so that it covered all the neurocages. The samples were

then incubated for an hour in a humidified (37 °C and 5 % $CO_2$ atmosphere) incubator (RS Biotech Laboratory Equipment Ltd, Irvine, UK).

A cell suspension was prepared with TrypLE using one well of neurospheres and 0NDM as the medium. After incubation, the neurocages were again located on the glass and the cells were seeded onto them with a 1 ml syringe and with a needle with a diameter of 330 µm. As the needle was much larger than the neurocages, the cell suspension was applied either with the droplet method or by injecting the suspension into the medium. The droplet method was used with sample OA2 and the injection method with OA1. After the application of the cells onto sample OA2, the Petri dish was filled with 2 ml of 0NDM. The Petri dish with sample OA1 was first filled with 2 ml of 0NDM, after which the needle was carefully placed on top of the neurocages and the cell suspension was gently injected into the medium. After seeding, the samples were imaged and photographed with an Olympus IX51 microscope (Olympus Corporation, Tokyo, Japan) and stored in the incubator. The software used in imaging and photography was TILLvisION (TILL Photonics GmbH, Munich, Germany).

After the first phase of experiment 1, the employed methods were evaluated. Samples OA3, OA4 and OA5 were then treated as OA1, except that the cell suspension used was prepared using two wells of neurospheres. This was done to obtain a denser cell suspension and thus increase the probability of the cell descending into the neurocages.

Samples OA1 and OA2 were cultured for a total of eight days and samples OA3, OA4 and OA5 for a total of seven days. The medium was changed three times a week. At each medium change, 1 ml of the medium was removed and 1.5 ml of fresh 5+NDM was added due to the evaporation of the medium in the incubator. The cells were also imaged and photographed before every medium change to observe the growth of the cells.

After eight days in culture, samples OA1 and OA2 were fixed to see whether the neurocages suffered from the fixing process. The medium was removed from the samples, which were then immersed in 4 % paraformaldehyde (PFA, Sigma-Aldrich, Saint Louis, USA). The samples were incubated in room temperature for 20 min, after which the PFA was removed and 2-3 ml of DPBS was added. The fixed samples were imaged and photographed, and afterwards sealed with Parafilm M (Pechiney Plastic Packaging Company, Chicago, USA) and stored in 4 °C.

### 5.3.2. Results and discussion

After the application of laminin, some of the nodes had air bubbles in them. This was thought to be due to the very small size of the nodes, which caused the surface tension of the laminin solution to prevent the solution from entering the nodes. However, as the samples were incubated, the air bubbles disappeared and all the nodes seemed to contain laminin.

The method of cell suspension application used with sample OA2 resulted in only a few cells in the nodes. The method used with sample OA1 seemed more promising,

though the number of cells in the nodes was still insubstantial. After five days in culture, it was found that all the cells in the samples had died. Figure 8 shows some of the neurocages on sample OA1 right after the application of the cell suspension and after five days in culture.

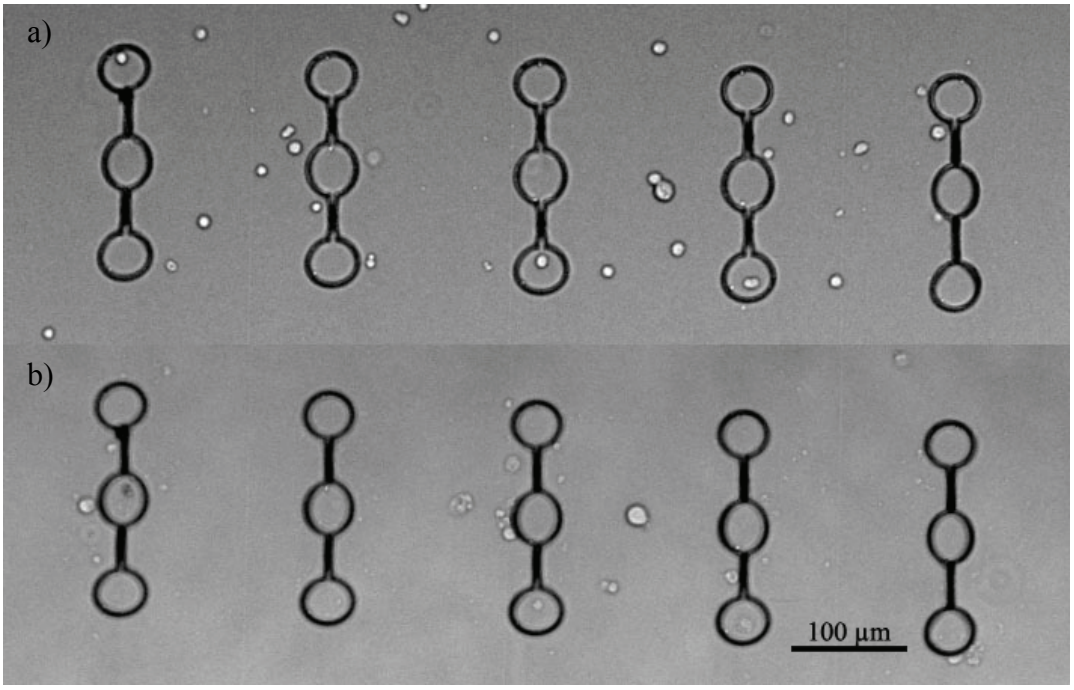



***Figure 8.*** *The neurocages on sample OA1 a) after the application of cells and b) after 5 days in culture.*

Samples OA1 and OA2 were fixed in order to study the effect of PFA on the neurocages, although there were only a few living cells in the samples. After the procedure, the samples were examined under a microscope and it was found that the neurocages were intact and still attached to the microscope glass surface. Some of the fixed neurocages on sample OA1 are shown in Figure 9.

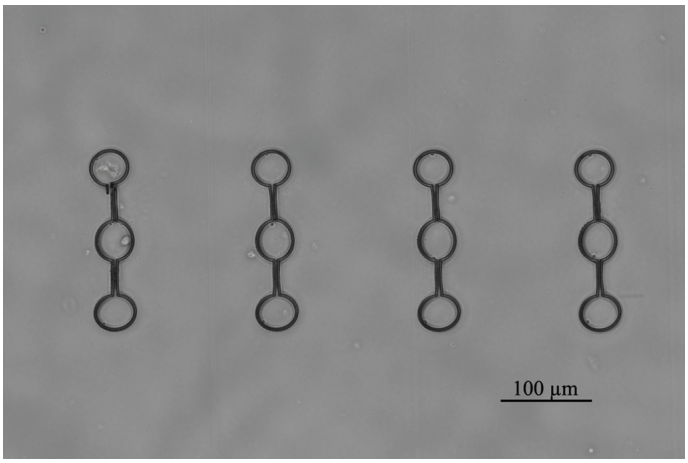



***Figure 9.*** *The neurocages on sample OA1 after PFA fixation.*

Because the method of cell suspension application used with sample OA1 was found better than the one used with sample OA2, samples OA3, OA4 and OA5 were treated as sample OA1, but with a denser cell suspension. The samples seemed very promising right after the application of laminin and cell suspension. Later, the live imaging of the samples showed that some cells had indeed attached to the glass inside the neurocages. However, after one week of culturing, all the cells in samples OA3, OA4 and OA5 had died. The cell death is illustrated in Figure 10, which shows a neurocage on sample OA3 right after the application of the cell suspension and after three days in culture.

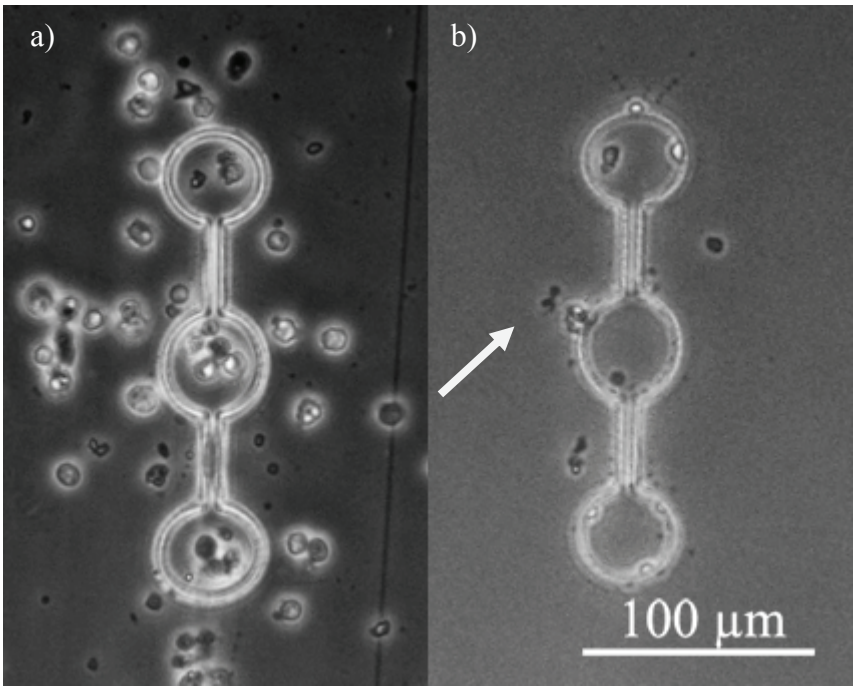



***Figure 10.*** *A neurocage on the sample OA4 a) after the application of cells and b) after 7 days in culture. Cell debris is pointed with a white arrow.*

As can be seen from Figure 10, there were very few live cells in the samples even after three days in culture. There were also some black cell fragments in the samples, as indicated by the white arrow in Figure 10 b). It should be noted that the neurocages in Figure 10 might not be the same neurocage, because identifying the neurocages was very difficult with the 20x objective used in the imaging.

A possible reason to the massive cell death was the fact that the samples might not have been completely immersed in medium during the cell culture. As the well area in the microscope slides containing the neurocages was surrounded by PTFE coating, the PTFE might have prevented medium exchange in the well if the glass slide was only partly immersed. Thus, the cells may have used all nutrients from the small volume of medium available and died of starvation. Another possible reason was that the neurocages released chemicals, monomers for example, which were toxic to neurons. Therefore for the next experiment, it was necessary to fabricate neurocages with various pre- and postfabrication methods and to study the cell viability with these different samples.

## 5.4. Experiment 2.1

The aim of the second experiment was to test the effect of the various neurocage pre- and postfabrication methods on the viability of the cells cultured with the neurocages.

### 5.4.1. Materials and methods

Experiment 2 was performed similarly to experiment 1. The first part of the experiment tested the various optional neurocage pre- and postfabrication methods described in Chapter 5.1. Five pairs of duplicate samples were used: OA6 and OA7 were fabricated as before, OA8 and OA9 were soaked in DPBS, OA9 and OA10 had been coated with 3-(trimethoxysilyl)propyl methacrylate before neurocage fabrication, OA12 and OA13 were baked and subsequently treated with UV light, and OA14 and OA15 were baked, treated with UV light and soaked in DPBS (see details in Chapter 5.1). The samples contained 9-12 neurocages each.

The laminin solution was applied onto the neurocages with the droplet method as in experiment 1. A cell suspension was prepared with TrypLE using four wells of neurospheres and 5+NDM as the medium. The cell suspension was injected into the medium as with sample OA1 in experiment 1 and the samples were incubated for an hour in the incubator before imaging and photography. After the imaging, the samples were stored in the incubator.

Samples OA6, OA9, OA10, OA13 and OA14 were cultured for a total of nine days and samples OA7, OA8, OA11, OA12 and OA15 for a total of fifteen days. The medium was changed and the samples were imaged and photographed as in experiment 1. After nine days in culture, samples OA6, OA9, OA10, OA13 and OA14 were fixed to prepare them for immunocytochemical staining. The fixing was performed as in experiment 1.

Two kinds of fluorescence staining procedures were performed on the samples. The viability of the cells was studied with live/dead staining, in which live and dead cells can be identified with fluorescent labels. Neuronal cells and astrocytes were identified with immunocytochemical staining, in which specific, cell type dependent proteins can be labelled and visualised with fluorescence. The programs used for the imaging were DP Controller and DP Manager (both from Olympus Corporation, Tokyo, Japan).

Samples OA7, OA8, OA11, OA12 and OA15 were live/dead stained. A commercial LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes, Invitrogen, Eugene, USA), which stained live cells with a green label and dead cells with a red label, was used for the staining. The medium was removed from the samples and 2 ml of 0NDM containing 0.1 µM of calcein AM and 0.5 µM of ethidium homodimer-1 was added. The samples were incubated for 30 minutes in the dark at room temperature, after which they were immediately fluorescence imaged and photographed. Live cells fluoresced in green and dead cells in read.

Samples OA6, OA9, OA10, OA13 and OA14 were immunocytochemically stained. The DPBS was removed and the samples were washed with fresh DPBS. Each sample was then blocked by adding 2 ml of a solution containing 10 % of normal donkey serum, 0.1 % of Triton-X and 1 % of bovine serum albumin (BSA) (all from Sigma-Aldrich, Saint Louis, USA) in DPBS and incubated 45 minutes at room temperature. After incubation, the samples were washed once with the primary antibody solution containing 1 % of normal donkey serum, 0.1 % of Triton-X and 1 % of BSA in DPBS. Primary antibodies against MAP-2 (anti-human MAP-2 rabbit IgG, 1:600, Millipore, Billerica, USA) and glial fibrillary acidic protein (anti-human GFAP sheep IgG, R&D Systems, Minneapolis, USA) were added to the solution, 2 ml was added to each sample and the samples were incubated at 4 °C overnight. After incubation, the samples were washed three times with the secondary antibody solution containing 1 % of BSA in DPBS. The secondary antibodies Alexa 488 donkey anti-sheep and Alexa 568 goat anti-rabbit (both 1:400, Invitrogen, Eugene, USA) were added to the solution, 2 ml of the secondary antibody solution was added to each sample and the samples were incubated one hour in the dark at room temperature. The samples were then washed three times with DPBS and two times with a phosphate buffer (pH 7; 0.1 M; Sigma-Aldrich, Saint Louis, USA). The samples were left to dry, after which they were mounted with Vectashield Mounting Medium for Fluorescence with 4’,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, USA) and immediately imaged and photographed. The neuronal cells on the samples fluoresced in red and astrocytes in green. Furthermore, all the cell nuclei fluoresced in blue.

### 5.4.2. Results and discussion

After the application of the cell suspension it was found that there was a varying amount of cells on the samples. However, no distinctive differences in the number of cells were found. There were already a few attached cells inside some nodes and many of the samples seemed very promising. Although the overall number of cells inside the nodes

was small, no additional cell suspension was added to the samples to prevent the already attached cells from detaching.

The growth of the cells was followed by imaging all the samples after two, six and nine days in culture. From the live images it was found that there were large differences between the duplicate samples, which might have been a result of inconsistent quality of the cell suspension application method. In general, the cells freely migrated to fill in the whole laminin-coated region of the microscope glass slides. Some of the samples were found to contain primarily neuronal cells (especially samples OA6, OA7, OA8 and OA9), while others were found to contain more cells with astrocyte morphology.

The cell growth in individual neurocages was followed by compiling series of images showing the same neurocage on the same sample at various time points (day 0, day 2, day 6 and day 9). As the neurocages were not numbered at this point of the study, identifying the individual structures was very difficult. With the 20x objective, it was found impossible to reliably identify an individual structure from the live images of various days. Hence, the series of images were all gathered from images taken with the 10x objective, although the neurocage structures appeared quite small with this magnification. The series of images from samples OA7, OA9, OA10, OA13 and OA14 are shown in Figures 11-15.

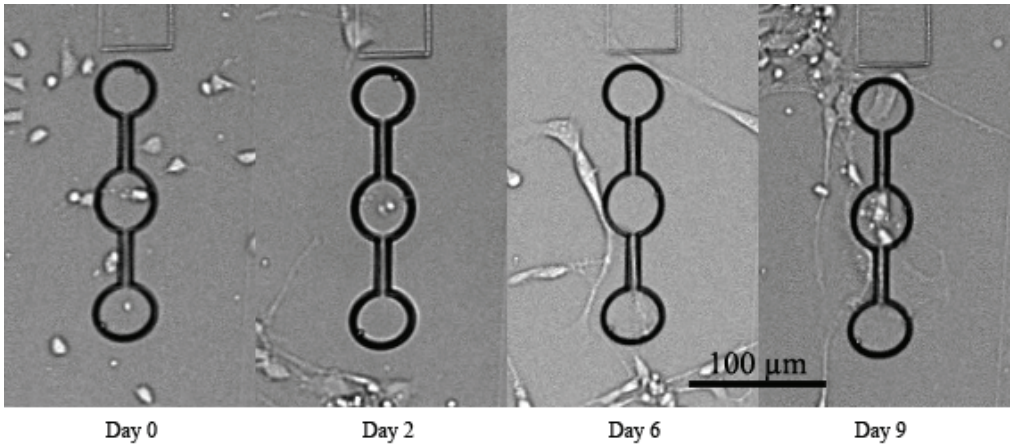



***Figure 11.*** *An individual neurocage on sample OA7 at various time points.*

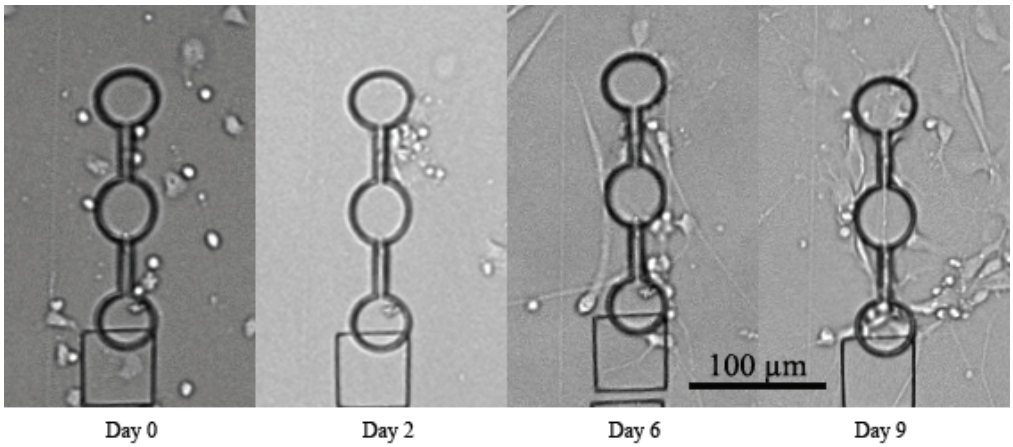



***Figure 12.*** *An individual neurocage on sample OA9 at various time points.*

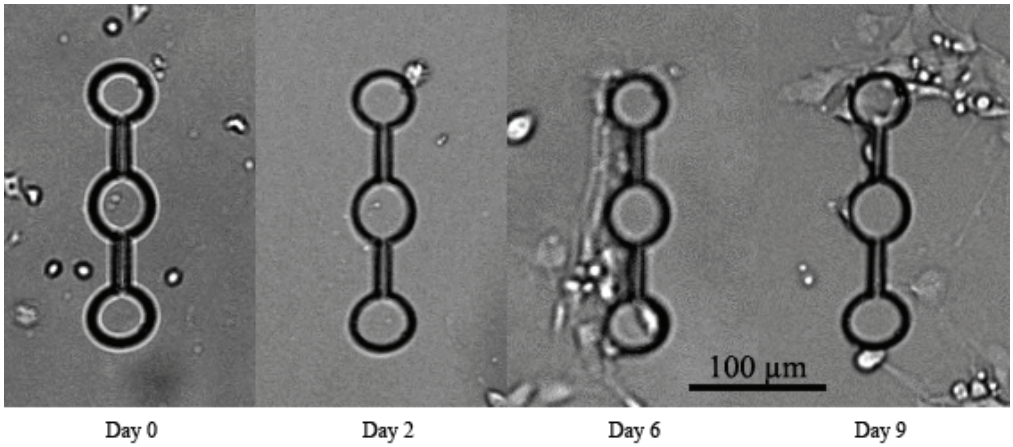



***Figure 13.*** *An individual neurocage on sample OA10 at various time points.*

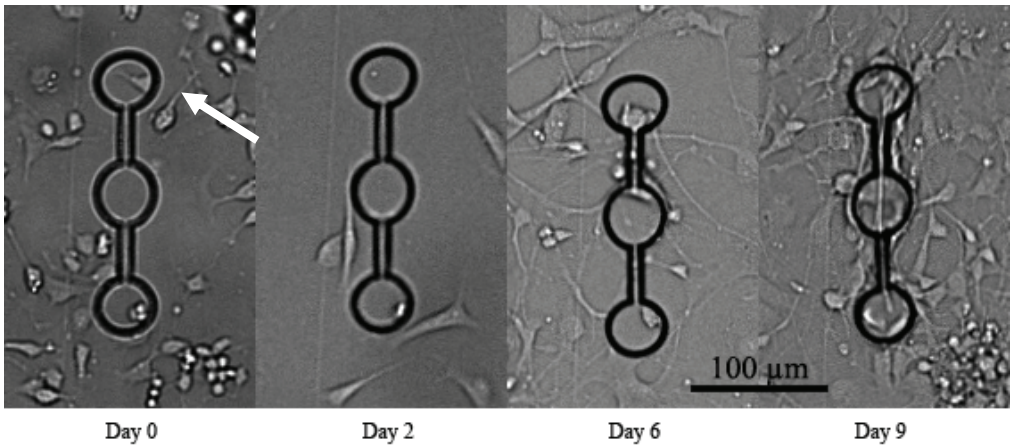



***Figure 14.*** *An individual neurocage on sample OA13 at various time points. A cell disappearing from the neurocage is pointed with a white arrow.*

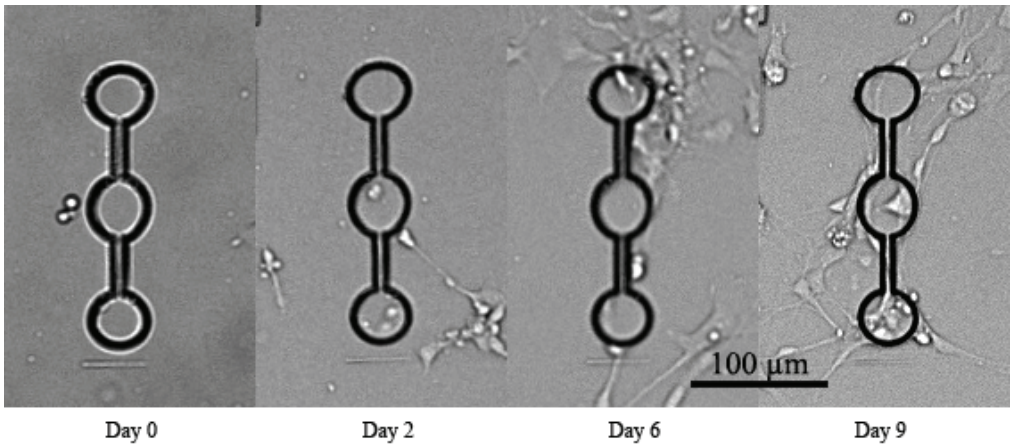



***Figure 15.*** *An individual neurocage on sample OA14 at various time points.*

It was found out that the cells grew freely in and out of the neurocages during the nine days in culture. It was not certain whether the cells initially located inside the neurocages migrated out of them or died. For example, the cell indicated by a white arrow in the first image of Figure 14 was no longer inside the neurocage two days later. It was very promising to notice that the cells evidently started to grow towards the neurocages and even inside them after the first few days in culture. This phenomenon is shown in Figures 11-14, where the cells can be seen to gather around and inside the neurocages in the images corresponding to six and nine days in culture. Even more promising was the fact that a possible neurites could be seen to extend inside the neurocages, as shown in the last images of Figure 12 and Figure 14.

The identity and viability of the cells located around and inside the neurocages was further studied by two fluorescence staining procedures. One duplicate of each sample pair was live/dead stained and the other immunocytochemically stained to see whether there was a difference in the viability or the phenotype of the cells present in the samples treated with different pre- and postfabrication methods. In Figures 16-20 both the live/dead and immunocytochemically stained images of a neurocage from each duplicate pair are shown.

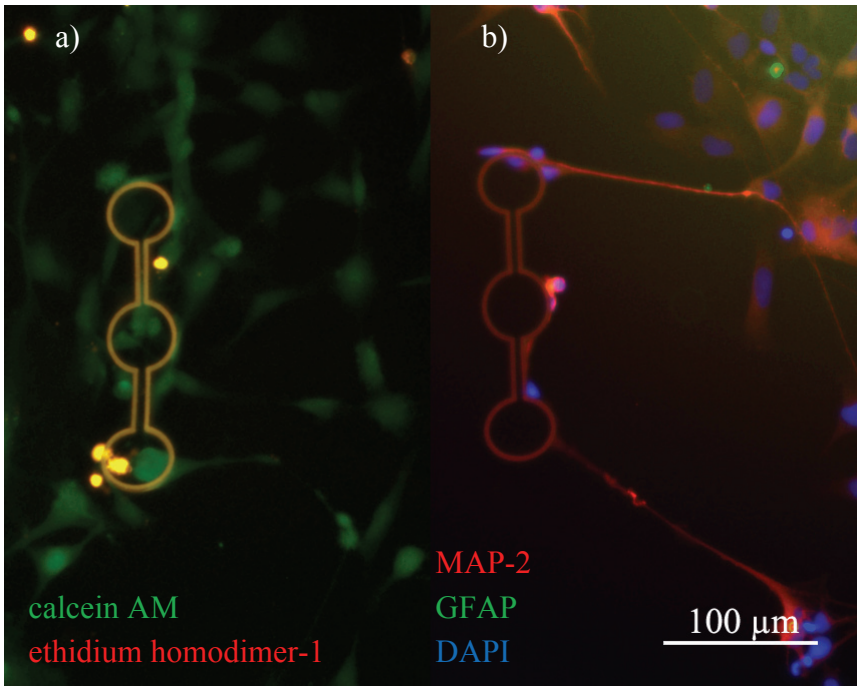



***Figure 16.*** *a) A live/dead stained neurocage from sample OA7. b) An immunocytochemically stained neurocage from sample OA6.*

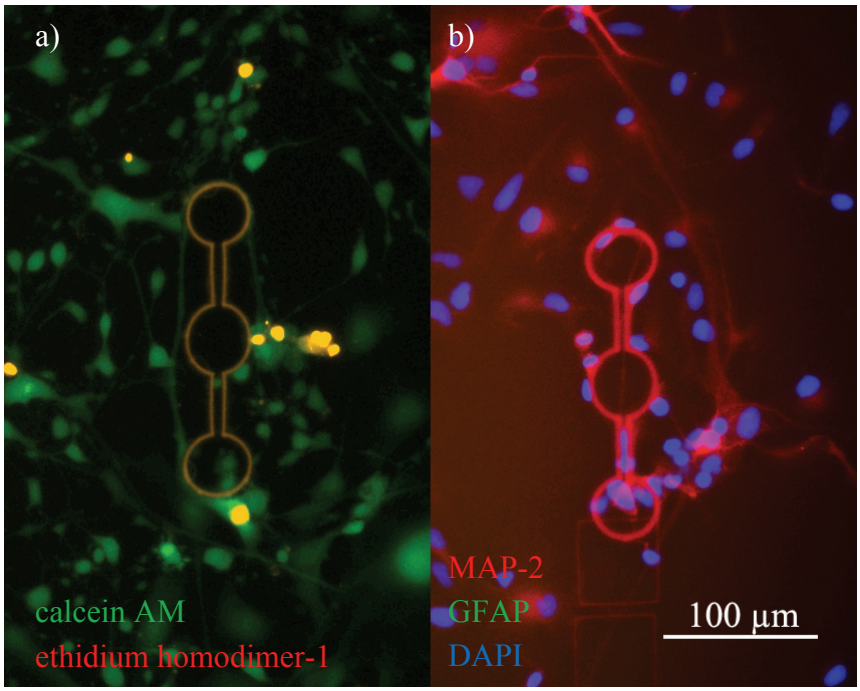



***Figure 17.*** *a) A live/dead stained neurocage from sample OA8. b) An immunocytochemically stained neurocage from sample OA9.*

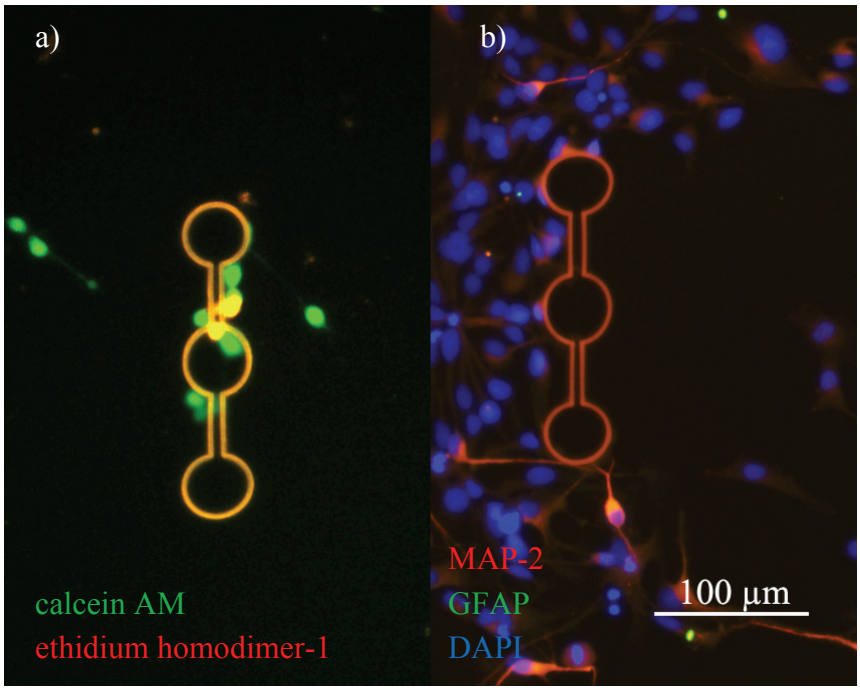



***Figure 18.*** *a) A live/dead stained neurocage from sample OA11. b) An immunocytochemically stained neurocage from sample OA10.*

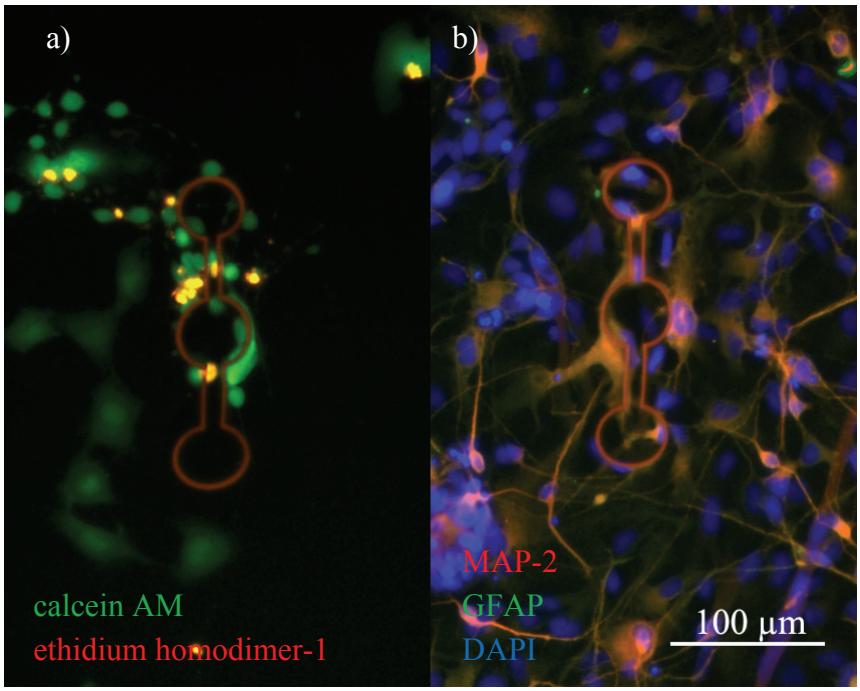



***Figure 19.*** *a) A live/dead stained neurocage from sample OA12. b) An immunocytochemically stained neurocage from sample OA13.*

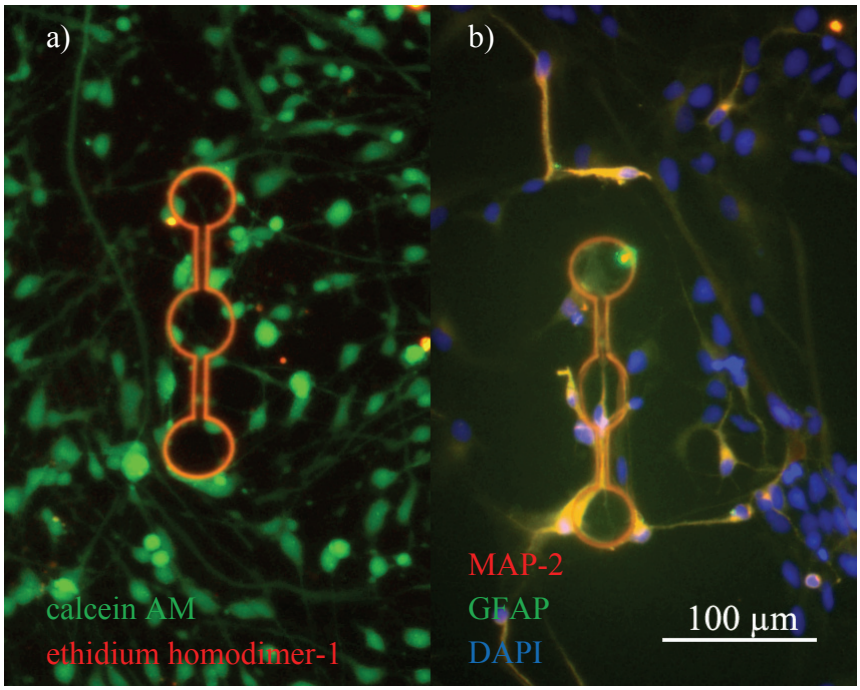



***Figure 20.*** *a) A live/dead stained neurocage from sample OA15. b) An immunocytochemically stained neurocage from sample OA14.*

Although the neurocages on the left and right side of Figures 16-20 were not from the same samples, they represented duplicates that had been fabricated by the same method. As all the samples were cultured in parallel, possible differences in cell viability or identity can be thought to result from the different neurocage pre- and postfabrication methods. However, as can be seen from Figures 16-20, there is very little difference between the five pairs of samples. The cells plated on all the differently fabricated neurocages were viable after 15 days in culture and many of the cells attached to the neurocages were MAP-2-positive, indicating that they were neurons. Hence, the neurocage fabrication method did not alter cell viability and the neurocages could be fabricated for the subsequent experiments by the simplest and easiest method.

As the cells could migrate freely in and out of the neurocages, the neuron confining properties of the neurocages were found to be insufficient. However, as the cells that eventually migrated into the neurocages readily extended their neurites along the neurocage tunnels, the neurite guidance properties of the neurocages were already very good. Therefore for the next experiment, it was necessary to specifically render the insides of the neurocages neuron-attractive to attach the cells to these regions only. Furthermore, as the neurons outside the neurocages would not survive in such an environment, it was necessary to make the cell suspension application method more accurate to seed enough cells into the neurocages.

## 5.5. Experiment 2.2

Because there was a need to render just the insides of the neurocages neuron-adhesive, the feasibility of two-photon polymerising a protein or peptide layer to the bottom of the neurocage nodes was evaluated. For this purpose, the cell adhesion promoting properties of the peptide glycine-arginine-glycine-aspartate-serine (GRGDS) were tested and compared to laminin.

### 5.5.1. Materials and methods

In the second part of the experiment 2, the peptide sequence GRGDS was tested as a coating substrate with laminin as a reference. Instead of neurocage samples, two 12-well plates (Nunc, Thermo Fisher Scientific, Rochester, USA) were used. The wells of each plate were numbered as shown in Figure 21.

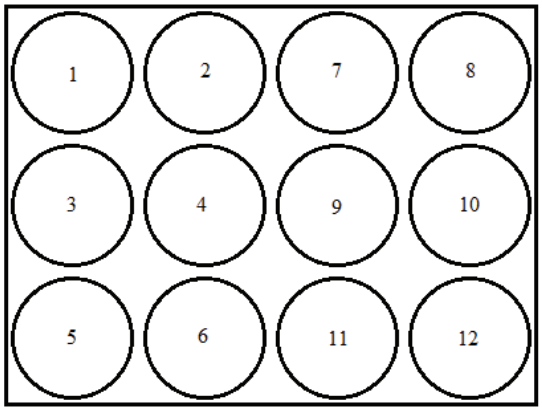



***Figure 21.*** *Graph of the 12-well plates used in experiment 2.2.*

The effect of 10 µg/ml of laminin and 10 µg/ml or 20 µg/ml of GRGDS (AnaSpec Inc, Fremont, USA) in sterile PBS (0.0249 mol/l $Na_2HPO_4$, 0.0055 mol/l $NaH_2PO_4 \cdot H_2O$, 0.1009 mol/l NaCl, prepared at the Department of Biomedical Engineering, Tampere University of Technology, Tampere, Finland) were tested on both plastic and glass surfaces. Six round glass coverslips with a diameter of 19 mm (Menzel GmbH, Braunschweig, Germany) were disinfected with ethanol and placed into the wells 1-6 of one plate. The plastic bottom plate was numbered as plate 1 and the glass bottom one as plate 2.

Two 10 µl drops of laminin were placed into the wells 1 and 2 on both plates. Similarly, a solution of 10 µg/ml of GRGDS was placed into the wells 3 and 4 and a solution of 20 µg/ml of GRGDS into the wells 5 and 6. The wells 7 and 8 of plate 1 were used as negative controls. Plate 2, however, did not have a negative control. The wells 9-12 were empty on both plates. The plates were incubated for two hours in the incubator.

A cell suspension was prepared with TrypLE using two wells of neurospheres and 5+NDM as the medium. After incubation, 1 ml of 5+NDM and 50-65 µl of the cell suspension were applied into the wells. After seeding, the plates were imaged and photographed, after which they were stored in the incubator.

The plates were cultured for a total of twelve days. The medium was changed three times a week and the plates were imaged and photographed before the changing of the medium as in the previous experiments. After twelve days in culture, the plates were fixed as in experiment 1 and stored in 4 °C.

### 5.5.2. Results and discussion

The plates were first imaged and photographed after two days in culture. At this stage, only a few cells were attached to the bottom of the wells. As the cells were further cultured, they slowly started to spread. Even so, after seven days in culture the number of cells in all the wells of plate 2 was small, although on laminin the cells were elongating processes and connecting to each other. On the contrary, the number of cells in the wells of plate 1 was larger and the cells started to spread soon after plating. However, the majority of the cells in the wells of plate 1 appeared to be flat epithelial-like cells instead of neurons or glial cells. The flat epithelial-like cells proliferated successfully even in the negative control wells of plate 1.

After twelve days in culture, neurons and glial cells could be found on both laminin- and GRGDS-coated surfaces on both plate 1 and plate 2. The fixed cells on both materials and on all three coatings are shown if Figures 22-27. In all the figures some cells with a neuronal morphology at the time of the imaging are pointed out with red arrows and some cells with a glial morphology with green arrows.

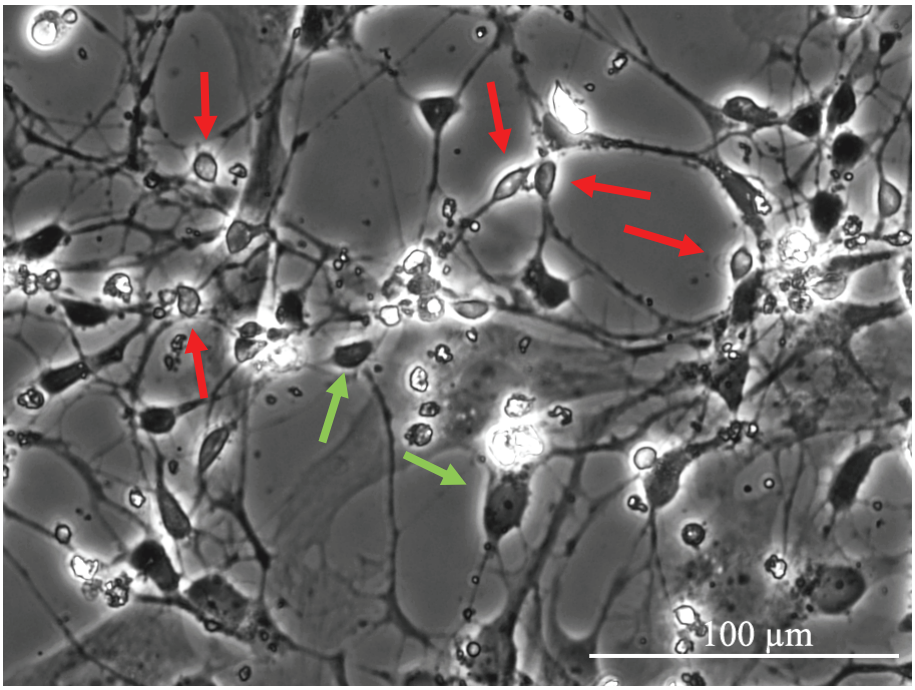



***Figure 22.*** *Cells growing on a glass substrate coated with a solution of 10 µg/ml of GRGDS peptide. Some cells with a neuronal morphology at the time of imaging are pointed out with red arrows and some cells with a glial morphology with green arrows.*

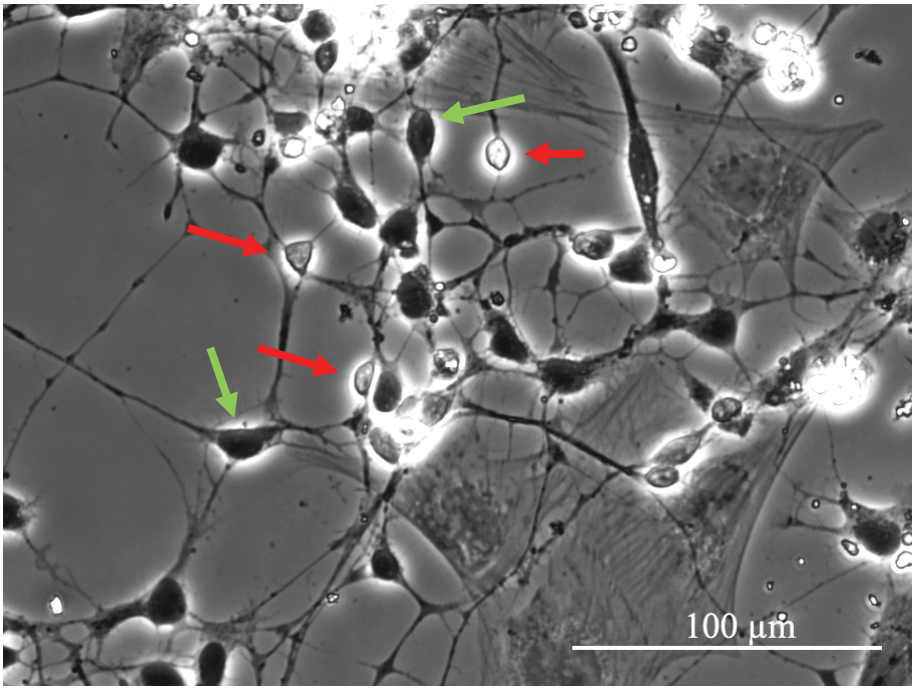



***Figure 23.*** *Cells growing on a glass substrate coated with a solution of 20 µg/ml of GRGDS peptide. Some cells with a neuronal morphology at the time of imaging are pointed out with red arrows and some cells with a glial morphology with green arrows.*

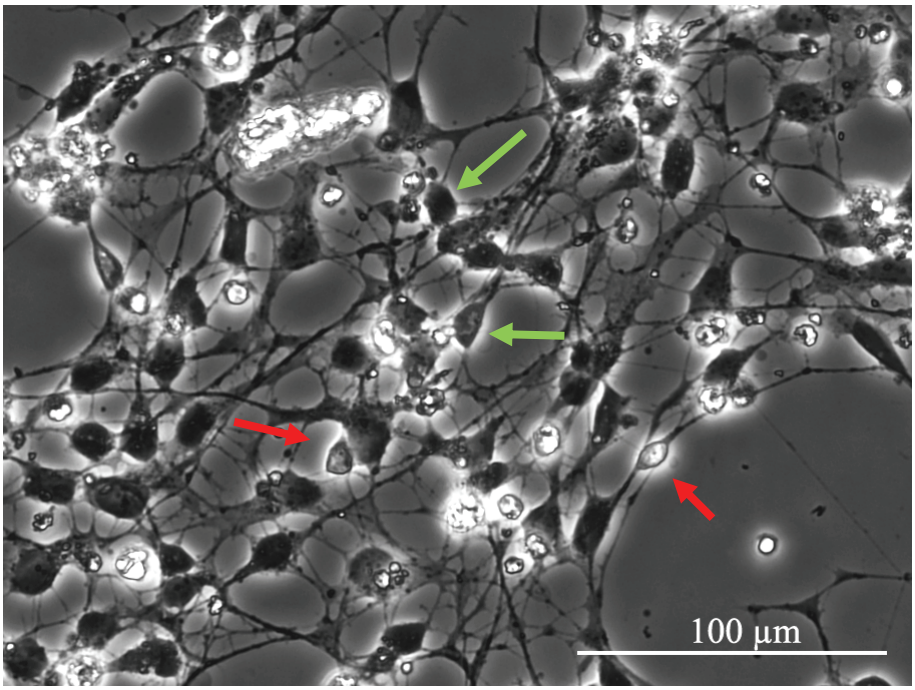



***Figure 24.*** *Cells growing on a glass substrate coated with a solution of 10 µg/ml of laminin. Some cells with a neuronal morphology at the time of imaging are pointed out with red arrows and some cells with a glial morphology with green arrows.*

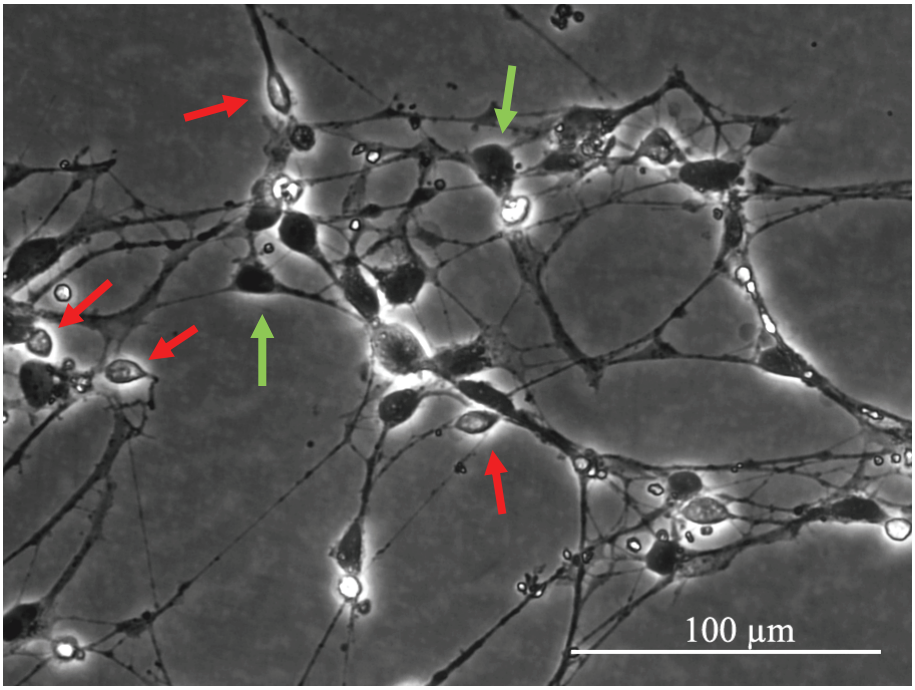



***Figure 25.*** *Cells growing on a plastic substrate coated with a solution of 10 µg/ml of GRGDS peptide. Some cells with a neuronal morphology at the time of imaging are pointed out with red arrows and some cells with a glial morphology with green arrows.*

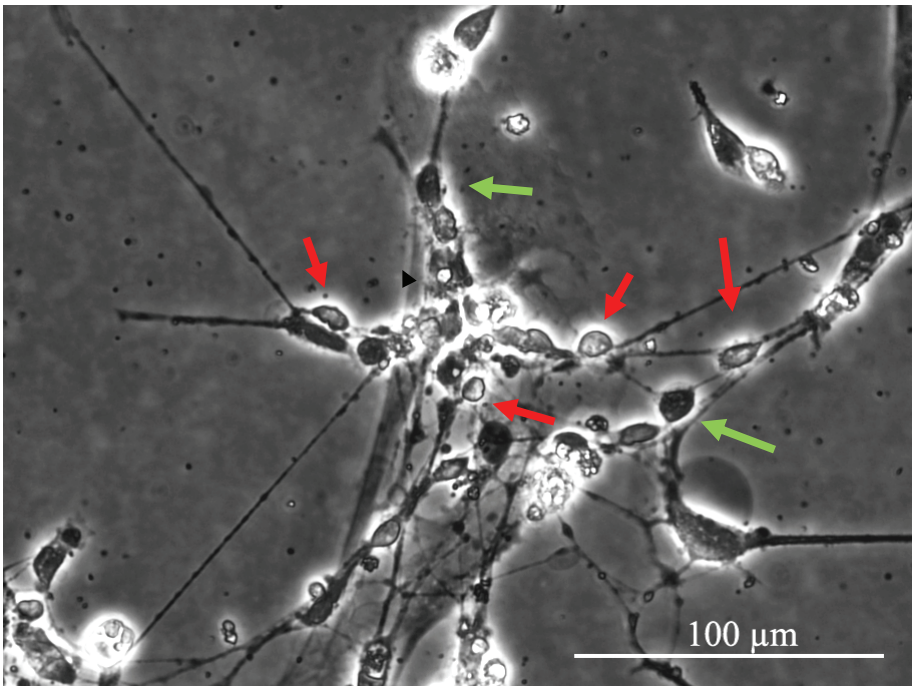



***Figure 26.*** *Cells growing on a plastic substrate coated with a solution of 20 µg/ml of GRGDS peptide. Some cells with a neuronal morphology at the time of imaging are pointed out with red arrows and some cells with a glial morphology with green arrows.*

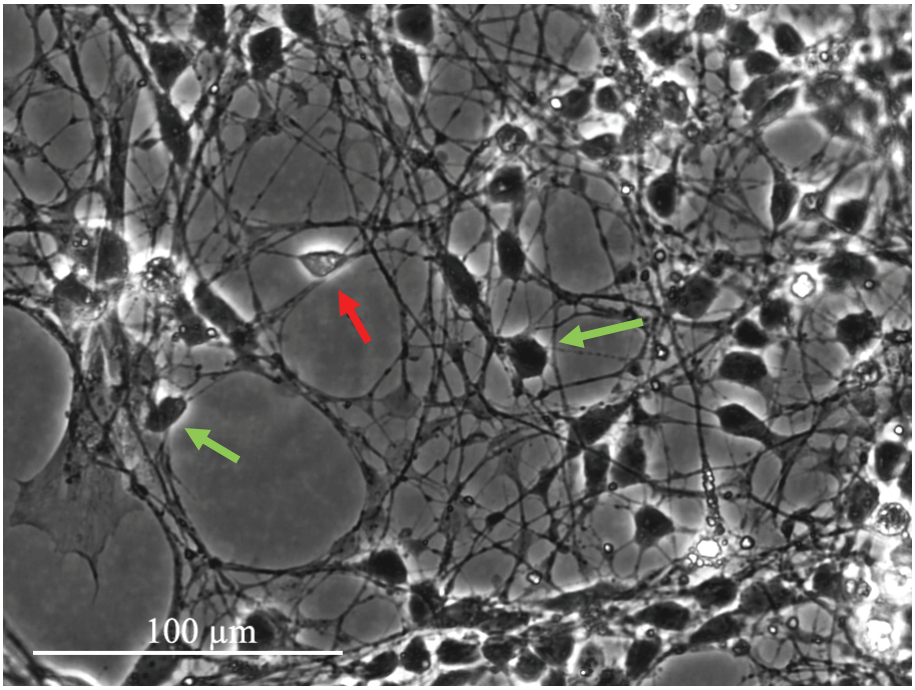



***Figure 27.*** *Cells growing on a plastic substrate coated with a solution of 10 µg/ml of laminin. Some cells with a neuronal morphology at the time of imaging are pointed out with red arrows and some cells with a glial morphology with green arrows.*

Figures 22-27 showed that neurons and glial cells proliferated successfully on both plastic and glass substrates and with both laminin and GRGDS coating. Although the protocol of this experiment was very simple, the cell-affinity of GRGDS was shown to be adequate to continue studies with this peptide.

## 5.6. Experiment 3

Because the studies of the two-photon polymerisation of GRGDS were still in very early stages, it was necessary to develop other methods to specifically coat the insides of the neurocages with a neuron-adhesive substrate. The aim of the experiment was to test a micromanipulator set-up in an attempt to inject the laminin solution and cell suspension with more precision.

### 5.6.1. Materials and methods

Experiment three was performed with the micromanipulator set-up SU1. SU1 consisted of an Olympus IX51 microscope (Olympus Corporation, Tokyo, Japan) connected to a PC, an anti-vibration table (Technical Manufacturing Company, Peabody, USA), two micromanipulators and a control device for the micromanipulators (all from Luigs & Neumann GmbH, Ratingen, Germany). For this study, a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) was attached onto the left-hand side micromanipulator. Additionally, a quartz glass capillary (Sutter Instrument Co, Novato, USA) pulled with Sutter Instrument P-2000 laser puller (Sutter Instrument Co, Novato, USA) was attached to the Hamilton syringe's needle with Blu-Tack® adhesive (Bostik, Paris, France). A patch clamp-device (Npi Electronic GmbH, Tamm, Germany) was attached to the right-hand side micromanipulator.

The samples tested were OA16, OA17, OA18 and OA19. Each sample contained nine numbered neurocages to ease the identification of the individual structures under the microscope. The Hamilton syringe with a glass capillary was used to apply the laminin solution onto the neurocages of OA16. The inner diameter of the capillary tip was approximately 20-50 µm and the Hamilton syringe was moved via the control panel of the micromanipulator. The neurocages were located under the microscope and the micromanipulator was carefully moved from one neurocage to another. When applying the laminin, the capillary tip was placed as close to a single node as possible and laminin solution was gently injected onto the structure. Generally, the diameter of the capillary tip was larger than that of an individual node (40 µm); hence the capillary tip could not be placed inside a node. Contrary to sample OA16, laminin was applied onto samples OA17, OA18 and OA19 with the droplet method as in experiment 1. After the application of laminin, all samples were incubated as in previous experiments.

A cell suspension was prepared with TrypLE from two wells of neurospheres with 5+NDM as the medium. After incubation, two different cell suspension application methods were tested. A method similar to the laminin application was used with sample OA18. In this method, the cell suspension was directly injected onto the neurocages.

With sample OA17, a drop of 5+NDM was first placed onto the glass so that it covered all the neurocages. The Hamilton syringe and attached glass capillary were then carefully placed over each neurocage and cell suspension was injected into the medium. Both methods required the use of several glass capillaries as the cell suspension easily blocked the capillary tips. After cell seeding, both samples were stored in the incubator.

The cell suspension used with sample OA19 was prepared with trypsin to produce a better disaggregation of the neurospheres in the suspension. Two wells of neurospheres and the 5+NDM medium were used. However, the cell suspension blocked most of the capillaries and no cells could be seeded into the neurocages.

### 5.6.2. Results and discussion

Although it was possible to pipette small droplets precisely with the Hamilton pipette, pressing of the piston generated vibration and movement in the glass capillary, which reduced the accuracy of the application of solutions. Furthermore, the piston needed to be pressed quite hard to push it down, but often the initial push was already too forceful and a large amount of solution became injected from the capillary tip. Therefore, the control of the droplet size and position was found rather difficult.

Even when the laminin solution was somewhat precisely injected onto a neurocage on sample 16, the extremely small volume of a single neurocage node brought up challenges. It was found that the water evaporated from the laminin suspension in seconds, leaving the crystallised laminin and other solutes into the nodes. This was not surprising as the volume of a node was in the range of picolitres and the laminin solution contained a substantial amount of salts and other solutes. After numerous attempts, it was found impossible to hold the solution in the nodes for a time period exceeding a few seconds.

An application method similar to the one used with the laminin solution was tested for the application of the cell suspension onto sample OA18. Unfortunately, the results were also similar to the ones found with laminin: the water in the cell suspension evaporated almost instantaneously, leaving behind the crystallised solutes and some cells. This phenomenon was not recorded because it occurred too fast for imaging. When a different approach of injecting the cell suspension into a droplet of medium was tested with sample OA17, the problems with the inadequate control of injection pressure re-emerged. The cells burst out of the capillary uncontrollably and spread into the medium droplet, finally settling to the outsides of the neurocage nodes. Additionally, the cell suspension contained different sized aggregates of cells, which often blocked the tips of the capillaries. To avoid the cell aggregates in the cell suspension, trypsin was tested as the disaggregating enzyme. However, the cell suspension treated with trypsin still contained the cell aggregates and blocked the rest of the capillary tips available.

The major obstacle of this experiment was the crystallisation of the laminin solution and the cell suspension. Therefore, for the next experiment it was necessary to find a way to prevent the crystallisation of the solutions. Additionally, the precision the

Hamilton syringe piston needed further optimisation to enable accurate injection of the solutions.

## 5.7. Experiment 4

The aim of the experiment was to find a way to retain enough moisture around the samples to prevent the minute amounts of the solution from crystallising. An idea of a water bath dish was invented and a prototype of the water bath dish was to be fabricated and tested.

### 5.7.1. Materials and methods

Experiment 4 was performed similarly to the experiment 3. Two samples, OA20 and OA21, were used. The samples were identical to those used in the experiment 3.

Novel water bath dishes were introduced in the experiment 4. Each dish was made by glueing the cover of a 35 mm Petri dish to the bottom of a 100 mm Falcon® Petri dish (Becton Dickinson Labware, Franklin Lakes, USA) as shown in Figure 29. The outer ring of the dish was filled with warm water and each sample was placed into the inner dish for the laminin and cell suspension application phase.

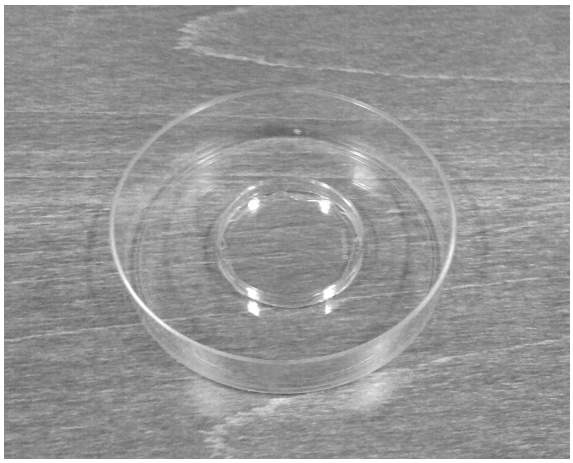


***Figure 29.*** *A water bath dish.*

Before the application of laminin, each water bath dish was covered with a 100 mm Petri dish cover that had a 1x1 cm opening for the glass capillary. The laminin solution was injected onto the neurocages with SU1 as in experiment 3, but with OA21, a solution of 100 µg/ml of laminin was used. After the application of laminin, the water bath dishes were covered with intact Petri dish covers and incubated; OA20 overnight and OA21 for an hour.

A cell suspension was prepared with trypsinisation using two wells of neurospheres and 5+NDM as the medium. The cell suspension was applied as in the experiment 3 with the exception that the samples were inside the water bath dishes. After the tests

with the Hamilton pipette, 10 µl of the cell suspension was injected thoroughly onto sample OA21. After the application of the cell suspension, 3 ml of 5+NDM was added onto each sample, the water bath dishes were covered as before and the samples were stored in the incubator.

On the following day, the sample OA21 was used to test whether the cells could be seeded with the patch clamp device on SU1. A cell suspension was prepared with trypsin using two wells of neurospheres and 5+NDM as the medium. Half of the medium on the samples was replaced with fresh 5+NDM, after which the cell suspension was injected into the medium as in experiment 1. The samples were incubated for an hour, after which the neurocages were located under the microscope as before. A capillary tip with inner diameter of either approximately 10 µm or 1-2 µm was connected to the patch clamp device and the pressure of the device was manually changed by various methods in an attempt to first capture the cells and then to free them above a neurocage. With the 10 µm capillary, the pressure of the device was controlled with a tube clamp (commonly used in intravenous infusion therapy to adjust the flow rate of liquids), a 1 ml syringe or the pressure regulator used in cell stretching experiments. The small capillaries were tested with the same pressure regulator and with an extra tube attached to a 60 ml syringe. In the latter test, the plunger of the syringe was removed and air was blown into or sucked from the barrel orally.

The samples were cultured for a total of 18 days. The medium was changed and the cells were imaged and photographed as described previously. After 18 days the samples were fixed similarly to the previous experiments and stored in 4 °C.

### 5.7.2. Results and discussion

Due to the utilisation of the water bath dishes, small droplets of the cell suspension stayed in the liquid state on the samples. However, the difficulties with the injecting accuracy still persisted and no cells could be injected into the neurocages. Although a larger volume of the cell suspension was also injected onto sample OA21, there were no living cells on the samples the next day.

The tests with the patch clamp device of SU1 further emphasised the limitations of human motor skills when controlling the aspiration and injection pressure. The cells could be successfully aspirated into the larger capillary by using either the 1 ml syringe or the pressure regulator. Furthermore, it was also possible to grab individual cells onto the tip of the smaller capillary with the pressure regulator. Unfortunately the manual control of the pressure formed a vacuum into the patch clamp device and cells were aspirated into the capillary even when the device was connected to an open piece of tubing. As a result, in most of the tests the cells could not be released from the capillary at all and even when they were released, they floated randomly in the medium.

The live imaging of the samples showed that there were some cells inside the neurocages but they were not attached to the glass even after several days in culture.

After 15 days in culture, there were a lot of flat epithelial-like cells on sample OA21. However, live neuronal cells were not observed at any stage of the experiment.

It became evident that it was not possible to inject laminin solution or cells into the neurocages when the pressure was controlled manually. Thus, for the next experiments it was necessary to use a set-up that included an automated pressure regulator. Even with an automated application system, it was obvious that the successive application steps for the laminin solution and the cell suspension into numerous individual neurocages would be troublesome and time-consuming. Therefore, it was also necessary to test whether the two components could be applied onto the neurocages in a single step.

## 5.8. Experiment 5

The aim of this experiment was to test whether the laminin solution and the cell suspension could be applied onto the neurocages in a single step. A single step would be very useful and make the sample handling faster and easier. If the solutions were to be applied onto each neurocage one by one, a single-step application method would be the only feasible option.

### 5.8.1. Materials and methods

Experiment 5 was very similar to the previous two experiments. The samples used were OA22 and OA23. Sample OA23 was put into a water bath dish as in experiment 4, whereas sample OA22 was tested without the water bath dish.

A cell suspension was prepared with trypsinisation using two wells of neurospheres and 5+NDM as the medium. The suspension was then filtered using a 50 µm filter (BD Biosciences, San Jose, USA) to include only single cells in the suspension and hence prevent the blockage of the capillaries. 1 ml of the suspension was separated and laminin was added to the suspension to prepare a final concentration of 20 µg/ml of laminin. This suspension was applied onto the neurocages as in the previous SU1 experiments. After the application of laminin, 2 ml of the medium 5+NDM was added onto the samples and they were stored in the incubator.

The samples were cultured for a total of eleven days. The medium was changed and the samples were imaged and photographed similarly to the previous experiments. After eleven days in culture, the samples were fixed as in the previous experiments and stored in 4 °C.

### 5.8.2. Results and discussion

The accuracy problems reported in the last two experiments still persisted in this experiment. As the piston of the Hamilton syringe was pressed, the capillary tip swayed and the cell suspension could not be accurately injected onto the neurocages. The swaying capillary tip also tore some of the neurocage structures off the glass slide. The

volume of the droplet injected also varied greatly and in the end all neurocages on the samples were either torn off or covered in a single large drop of cell suspension. Figure 30 shows the neurocage 2 on sample OA23 after a drop of the cell suspension has been injected onto the neurocage. The accuracy and droplet size were one of the best achieved, which illustrates the difficulty of accurately applying the solutions.

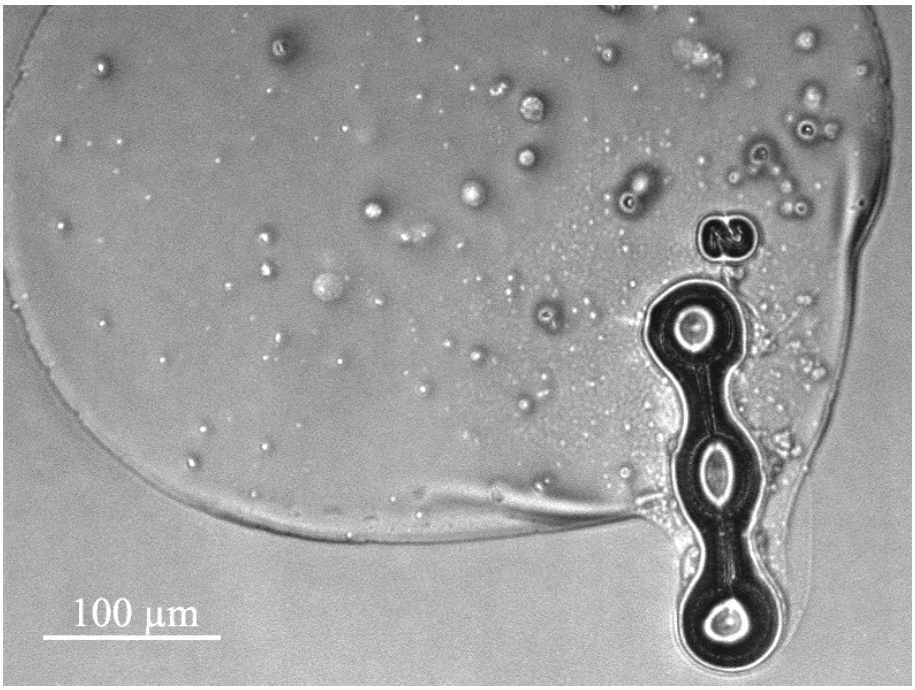



***Figure 30.*** *A neurocage on sample OA23 covered with the cell suspension.*

As the samples were imaged after five and eight days in culture, a lot of spherical cells were found floating on the medium. However, there were no cells attached to the glass on either of the samples. A possible reason to this was the fact that the laminin stock solution was diluted directly into the cell suspension. Another relevant factor was the use of trypsin as the disaggregating enzyme. Trypsin is very harsh on the cells and there was a strong possibility that many of the single cells in the suspension had died during the disaggregation treatment. The cell suspension was also filtered prior to the application onto the neurocages and the small cell aggregates were thus left out. This could explain the lack of attached cells, as the experiment protocol was otherwise very similar to that used in experiment 2, where a lot of attached and proliferating cells were observed. It was therefore necessary to closely observe the cells of the next experiment and discontinue the use of trypsin if low cell attachment was still observed.

## 5.9. Experiment 6

The aim of the experiment was to test the novel micromanipulator set-up SU2 for the application of the laminin solution. Because the set-up had not been used in a similar experiment before, the first task was to find suitable injection pressure and injection time to accurately fill the nodes of the neurocages.

### 5.9.1. Materials and methods

Experiment 6 was the first performed with the micromanipulator system SU2. SU2 consisted of a micromanipulator, a control device for the micromanipulator, a PC-controlled pressure regulation system (all developed by the Department of Automation Science and Engineering, Tampere University of Technology, Tampere, Finland) and a Nikon Eclipse TS100F inverted microscope (Nikon Corporation, Tokyo, Japan) connected to a PC. In this study, a capillary with a tip diameter of approximately 10 µm was attached to the micromanipulator.

The sample used was OA24, which was placed into a water bath dish for the application of laminin. Contrary to all the other samples, OA24 was not disinfected with ethanol prior to the experiment, because the aim of the experiment was to only test the novel set-up. For the same reason the laminin solution used in the experiment was not stored in a refrigerator contrary to the previously used solutions.

The capillary tip was filled with a pipette prior to its attachment to the micromanipulator. The sample was placed under the microscope and the neurocages were located. The micromanipulator was moved to a correct position near the neurocages, the water bath dish was filled with warm water and a cover with a novel, radial 5 mm wide gap was put onto the dish. The capillary tip was carefully moved from one node to another and pressure was applied to fill the nodes with the laminin solution. Various pressure settings were found to be successful, one of them being 150 mbar pressure and 4010 ms injection time. After the application of laminin, the water bath dish was covered with an intact Petri dish cover, the dish was sealed with Parafilm M and the sample was incubated overnight at room temperature.

A cell suspension was prepared with trypsinisation using two wells of neurospheres and 5+NDM as the medium. The suspension was also filtered as in the previous experiment. After incubation, the sample was placed to a 35 mm Petri dish and immersed in DPBS for an hour. The sample was again moved to a new Petri dish, 2 ml of the medium 5+NDM was added and the sample was incubated to remove any air bubbles from the nodes. The cell suspension was applied with a BD Microlance 3 needle (outer diameter 330 µm, Becton Dickinson S.A., Fraga, Spain) and a 1 ml syringe. The sample was placed under an Olympus CK2 microscope (Olympus Corporation, Tokyo, Japan) and the structures were located. A droplet of the suspension was injected onto the tip of the needle and carefully placed onto the surface of the medium atop the neurocages. After application, the cells were let to settle into the structures for about 15 minutes, after which the sample was stored in the incubator.

The sample was cultured for a total of seven days. The medium was changed three times a week and the neurocages were imaged and photographed as in the previous experiments. After seven days in culture, the sample was live/dead stained to study whether there were viable neurons on the sample. The protocol was essentially the same as that used in experiment 2 but a mixture of Dulbecco's Modified Eagle Medium and Nutrient Mixture F12 was used as the staining medium.

### 5.9.2. Results and discussion

Although the micromanipulator set-up SU2 was used in this experiment with very low expectations, the set-up was an immediate success in the application of the laminin solution. The three-dimensional navigation of the capillary was very easy contrary to the previously used micromanipulator set-up SU1. Furthermore, the accurate pressure regulation of set-up SU2 enabled the precise application of the laminin solution into the neurocages, as shown in Figure 31. A downside of the set-up was a possible blockage in the capillary tip, which easily led to increase of pressure inside the capillary and eventually burst the solution out and covered several neurocages with the solution. However, this could be avoided by checking the integrity of the capillary tip before use and by careful positioning of the tip.

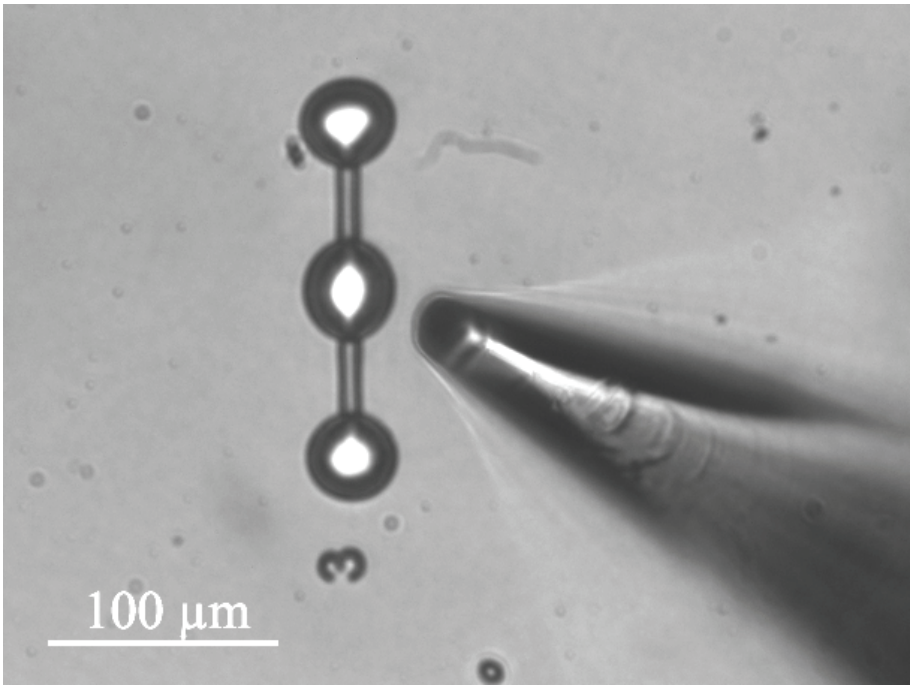



***Figure 31.*** *A neurocage on sample OA24 with the laminin solution inside the neurocage.*

Due to the successful application of the laminin solution, the sample was used in cell culture despite the fact that it had not been disinfected. In this experiment, a novel cell suspension application method was also tested. Contrary to previous experiments,

the cell suspension was not injected into the medium but a drop of the suspension was gently placed onto the surface of the medium. With this method the cells did not float randomly in the medium but descended quite accurately onto the glass surface below the application point. However, as the sample was imaged on the day following the application of the cell suspension, most of the initially descended cells had disappeared. It was possible that the 15 minute incubation after the application of the cell suspension was not enough and even the slightest movement caused the cells to float away. Another important factor was the storage of the laminin solution. Contrary to previous experiments, the laminin solution was stored at room temperature, which could have affected the cell-adhesive properties of the solution. After four days in culture, there were only a few attached cells on the sample and these cells resembled flat epithelial-like cells more than neuronal cells. However, after seven days in culture the live/dead staining showed that some viable neuronal cells were present on the sample. It was especially noteworthy that the neurons had extended their axons towards the neurocages, as shown in Figure 32.

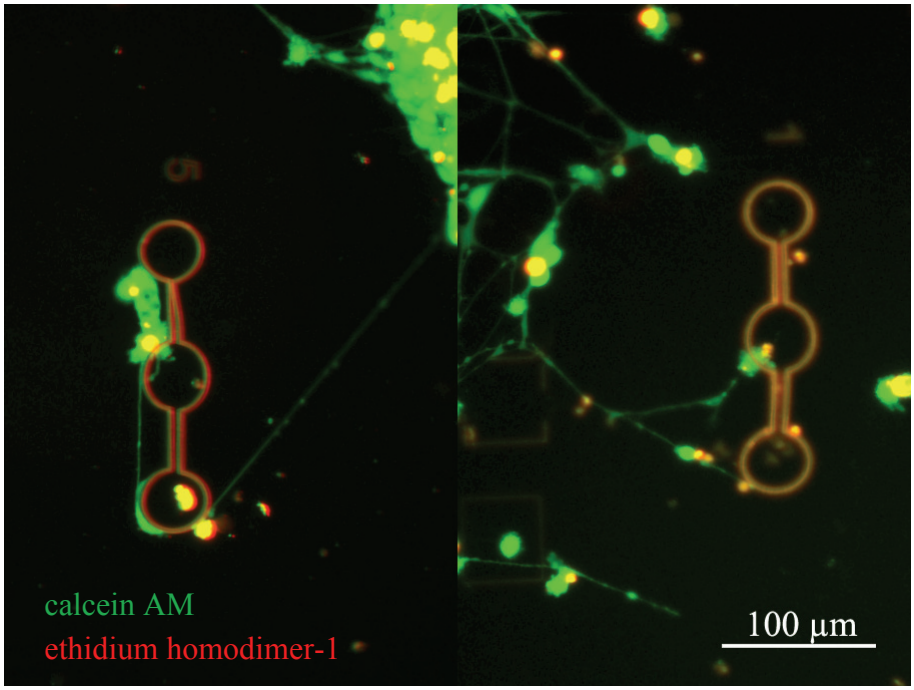



***Figure 32.*** *Neuronal cells extended their axons towards the neurocages on sample OA24.*

This experiment showed that the new micromanipulator set-up was very feasible for the application of the laminin solution. The novel method for the application of the cell suspension was also found relatively accurate, especially if the settling time for the cells could be slightly extended. As the methods for both application steps were now straightforward to execute, they could be utilised with the more challenging design B neurocages.

## 5.10. Experiment 7

The aim of the experiment was to test the optimised methods for the application of the laminin solution and the cell suspension with the design B neurocages. Contrary to the design A neurocages, all nodes in the design B neurocages were identical. The nodes also offered the cells three competing tunnels, which could be utilised to test the optimal dimensions of the neurocages for the extension of neurites.

### 5.10.1. Materials and methods

Experiment 7 was essentially the same as experiment 6, except that the neurocages used were of the design B. Each of the samples OB2, OB3, OB4 and OB5 contained four numbered neurocages. The samples were disinfected with ethanol as previously and placed into the water bath dishes. A solution of 50 µg/ml of laminin was applied as in the experiment 6. After the application of the laminin solution, the water bath dishes were covered with intact Petri dish covers, sealed with Parafilm M and incubated in a refrigerator overnight.

A cell suspension was prepared with trypsin using two wells of neurospheres and 0NDM as the medium and filtered as in the previous two experiments. After the incubation, the samples were immersed in DPBS and incubated to remove any air bubbles. The DPBS was removed, 3 ml of the medium 0NDM was added and the samples were again incubated to remove the rest of the bubbles. Some of the bubbles did not dissolve and before the application of the cell suspension, the structures of OB4 were carefully aspirated with a pipette, which tore the structures off the glass. The other samples were hence not touched. The cell suspension was applied as in experiment 6, after which the samples were stored in the incubator.

The cells were cultured for a total of eight days. The medium was changed and the samples were imaged and photographed as in the previous experiments. After eight days in culture, the samples were fixed as previously described and immunocytochemically stained. The staining protocol was essentially the same as that used in experiment 2. The primary antibodies used were anti-human MAP-2 rabbit IgG and anti-human GFAP sheep IgG. Contrary to experiment 2, the secondary antibodies used were Alexa 488 donkey anti-rabbit and Alexa 568 donkey anti-sheep (both 1:400, Invitrogen, Eugene, USA). Hence, in this experiment neuronal cells fluoresced in green and astrocytes in red.

### 5.10.2. Results and discussion

Although the laminin application step was identical to that used in the previous experiment, for some reason the laminin solution disappeared rapidly from the nodes. It was uncertain whether the solution evaporated but no crystallisation comparable to that in experiment 3 was observed. The laminin solution had to be added several times to each node and the application was stopped when an adequate amount of the nodes

contained the solution. It was speculated whether the disappearance of the solution was caused by the disinfection of the samples as such a phenomenon was not observed with sample OA24. It was possible that some ethanol had been absorbed into the neurocages during disinfection.

After four days in culture, there were aggregates of round cells on all the samples. On samples OB2 and OB5, the few attached cells had the morphology of flat epithelial-like cells. There were numerous cells inside the neurocages but they were not attached to the glass surface. On OB3, which was completely covered with the laminin solution, the majority of the region around the neurocages was covered with attached cells. The nodes of the neurocages, however, contained only round cells that were not attached to the surface. It was uncertain why the cells thrived outside the neurocages but did not adhere to the surface inside. After six days in culture, samples OB2 and OB5 still contained primarily flat epithelial-like cells and the areas around the neurocages on OB3 were largely covered by neuronal or glial cells and their processes, as shown in Figure 33. Figures 34, 35 and 36 illustrate the immunocytochemically stained samples OB2, OB3 and OB5, respectively.

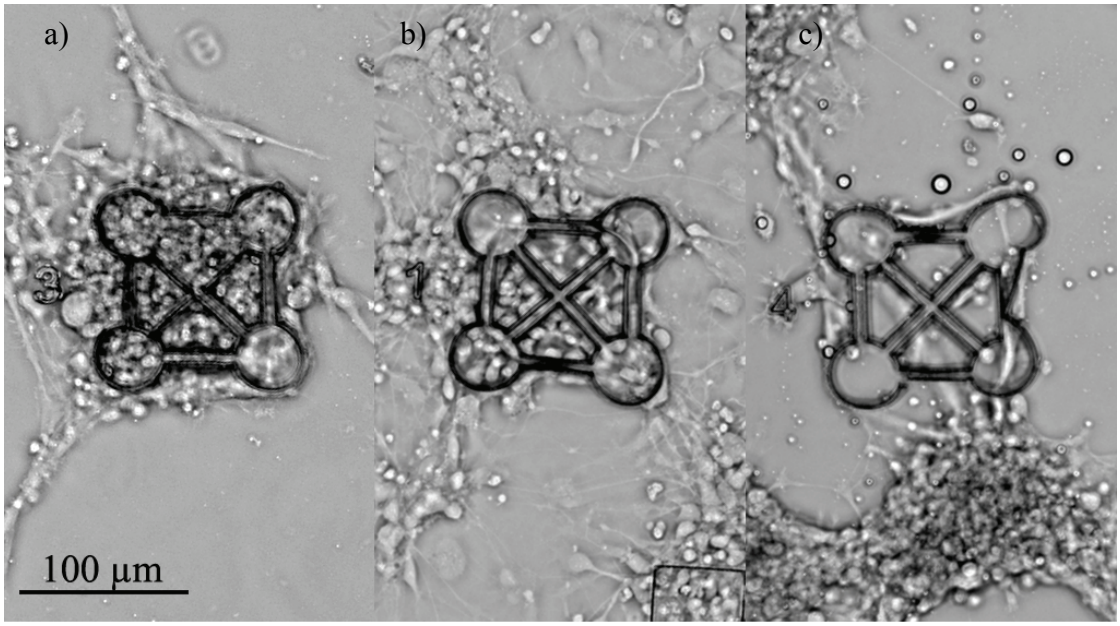



***Figure 33.*** *Neurocages on samples a) OB2, b) OB3 and c) OB5 after six days in culture.*

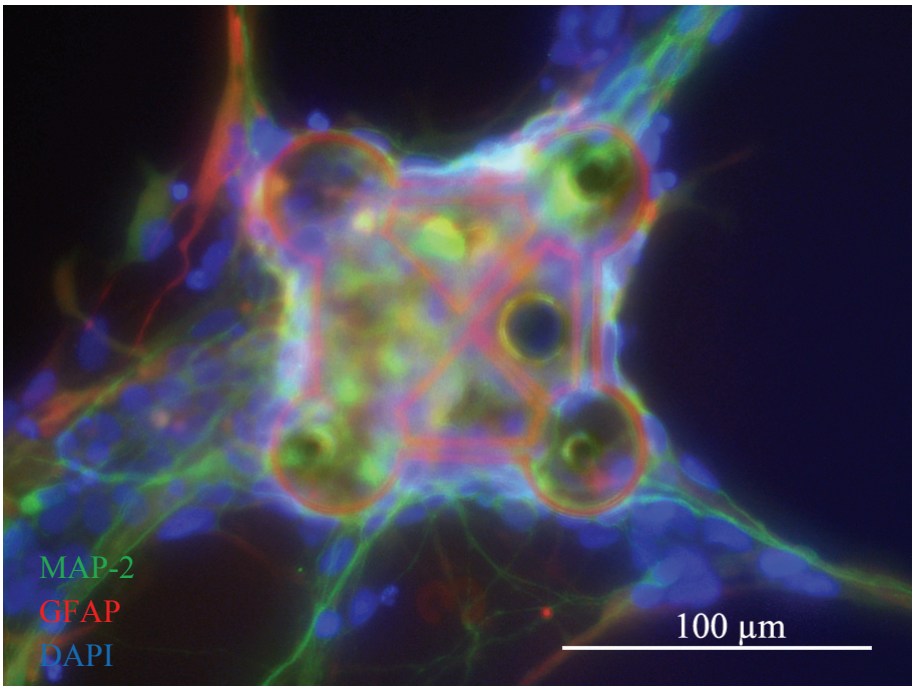



***Figure 34.*** *An immunocytochemically stained neurocage from sample OB2.*

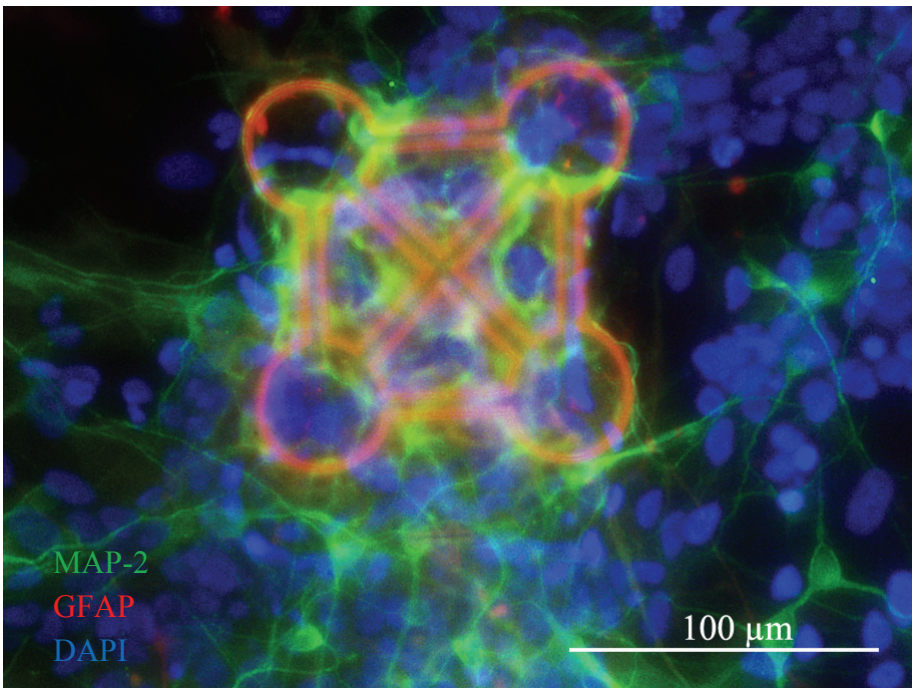



***Figure 35.*** *An immunocytochemically stained neurocage from sample OB3.*

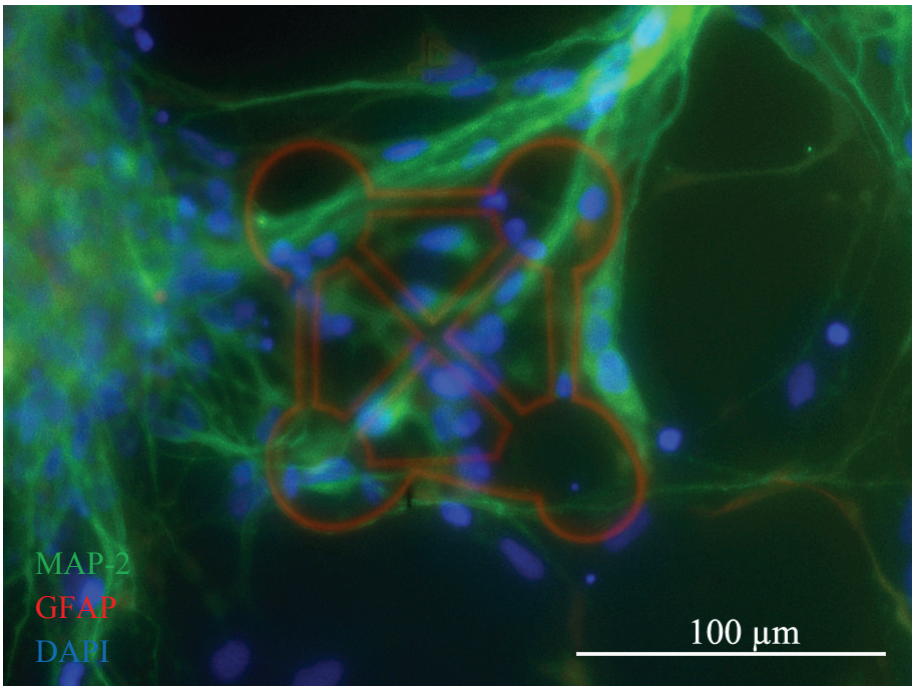



***Figure 36.*** *An immunocytochemically stained neurocage from sample OB5.*

After the immunocytochemical staining it was observed that there were viable neuronal cells on the samples and even entwined processes of several neurons on sample OB5. However, from Figure 34 to 36 it was not possible to determine whether the processes had migrated over the neurocage walls to grow on the bottom of the nodes or were continuously migrating on top of the neurocage walls.

Although there were some viable neurons on the samples of this and the previous experiment, the number of cells was substantially lower than that of experiment 2 and neurons generally did not adhere to the surface inside the neurocages. Because the precise application of the laminin solution inside the neurocages prevented neurons from effectively attaching to and proliferating on the regions outside the neurocages, it was possible that the overall small number of cells in the culture hindered the growth of the cells. It has been observed by Erickson and co-workers that a small neuronal population does not survive but needs a supporting neuron population to condition the medium [11]. In this study, it was proposed that a supporting neuron population could be substituted by adding some medium taken from another neuronal culture to the medium used with the samples. Such medium is called a conditioned medium, meaning that the neuronal cells cultured with it have secreted specific growth factors and other molecules into it. This method was tested in the next experiment.

## 5.11. Experiment 8

The aim of the experiment was to test whether the viability of the cells could be increased with the use of medium conditioned by another neuronal cell culture. It was also necessary to increase the general viability of the single cells in the suspension. Therefore, trypsin was discarded as the disaggregating enzyme and replaced with a filtering step to remove cell aggregates from the suspension.

### 5.11.1. Materials and methods

Experiment 8 was essentially the same as experiments 6 and 7. However, various optimising steps were taken to ensure the viability of the cells inside the neurocages. The samples used were OA25, OA26, OA27 and OA28, each of them containing six numbered neurocages.

The samples were disinfected with ethanol and immersed in DPBS for approximately 65 hours to ensure that no ethanol residues were absorbed into the neurocages. The preparation of the samples was essentially the same as in the previous two experiments, but the water used in the water baths was hot and it was added right before the application of the laminin. After the application of laminin onto the sample OA26, the capillary was changed from that used in the previous two experiments to one with a tip diameter of approximately 5 µm. At the same time, the pressure was shifted from 100 mbar to 150 mbar and the injection time was changed from 4010 ms to 6000 ms. After the application of laminin onto the sample OA27, further adjustments were made as slightly cooler water was used in the water bath. Laminin was applied onto OA28 and OA25 with the cooler water with excellent results. After the application of the laminin solution, intact covers were placed on the water bath dishes, the dishes were sealed with Parafilm M and incubated in a refrigerator overnight.

A cell suspension was prepared with TrypLE using two wells of neurospheres, 0NDM as the medium and by filtering the suspension. After the filtration, 1 mg/ml of the laminin solution was added to the suspension to a concentration of 20 µg/ml. After the incubation, the samples were immersed in DPBS and incubated in the incubator as before. DPBS was then removed and 2 ml of 0NDM was added to each sample. Additionally, 850 µl of the conditioned 0NDM was applied to each sample. The cell suspension was applied as in the previous two experiments, after which the samples were stored in the incubator.

The cells were cultured for a total of five days. The medium was changed three times a week. At each medium change 1.5 ml of medium was removed and 1 ml of fresh 5+NDM and 800 µl of conditioned 0NDM were added. Before each medium change the cells were imaged and photographed as in the previous experiments. After five days in culture the samples were immunocytochemically stained. The staining protocol was identical to that used in experiment 7.

### 5.11.2. Results and discussion

Although the application of the laminin solution had been very successful, there was no evident impact of the application success on the viability of cells on the samples. The laminin application step was most successful on samples OA25 and OA28, but after two days in culture samples OA25 and OA27 were the ones with the most attached cells. However, the cells on all the samples had formed elaborate networks after only two days in culture, which suggested that the use of the conditioned medium had a definite effect on the viability of the cells. The extensive growth of the cells further proposed that the success of the laminin application had little effect of the cell viability, as the cells proliferated successfully outside the neurocages even without laminin. Figures 37-40 show the growth of the cells inside and around a few individual neurocages of the samples after two and three days in culture.

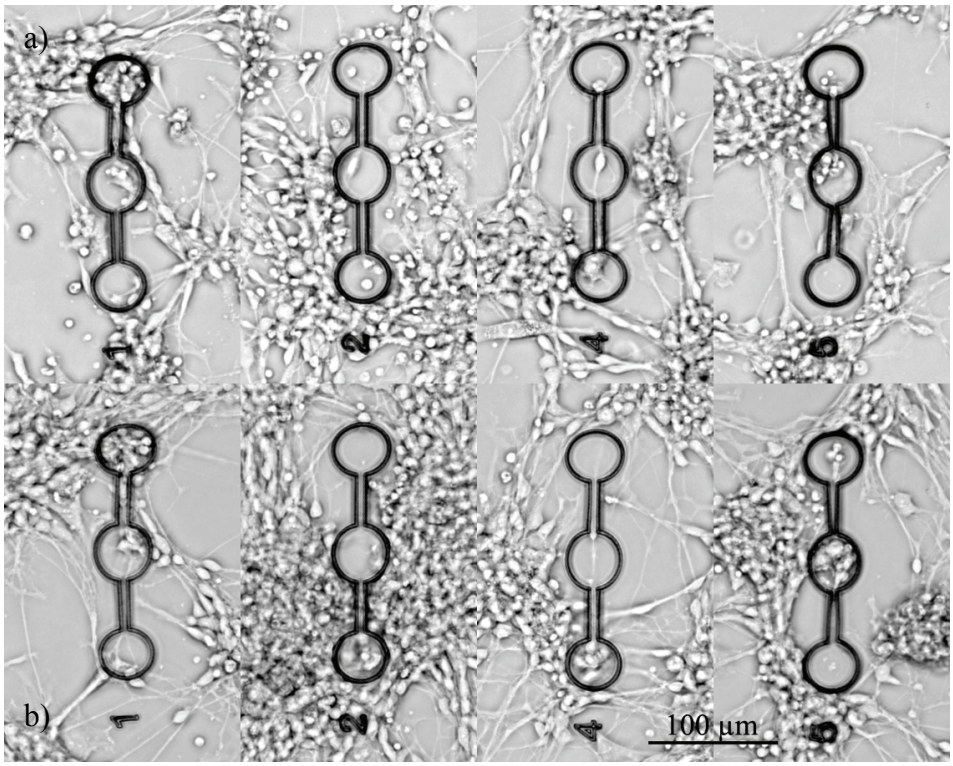



***Figure 37.*** *Individual neurocages on sample OA25 after a) two and b) three days in culture.*

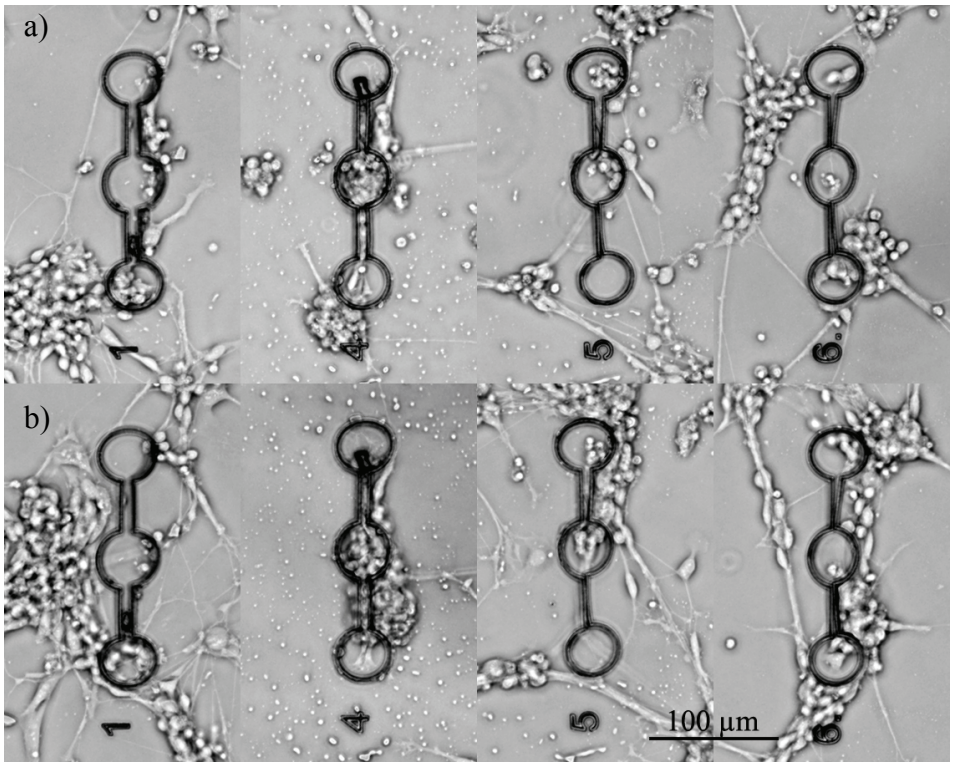



***Figure 38.*** *Individual neurocages on sample OA26 after a) two and b) three days in culture.*

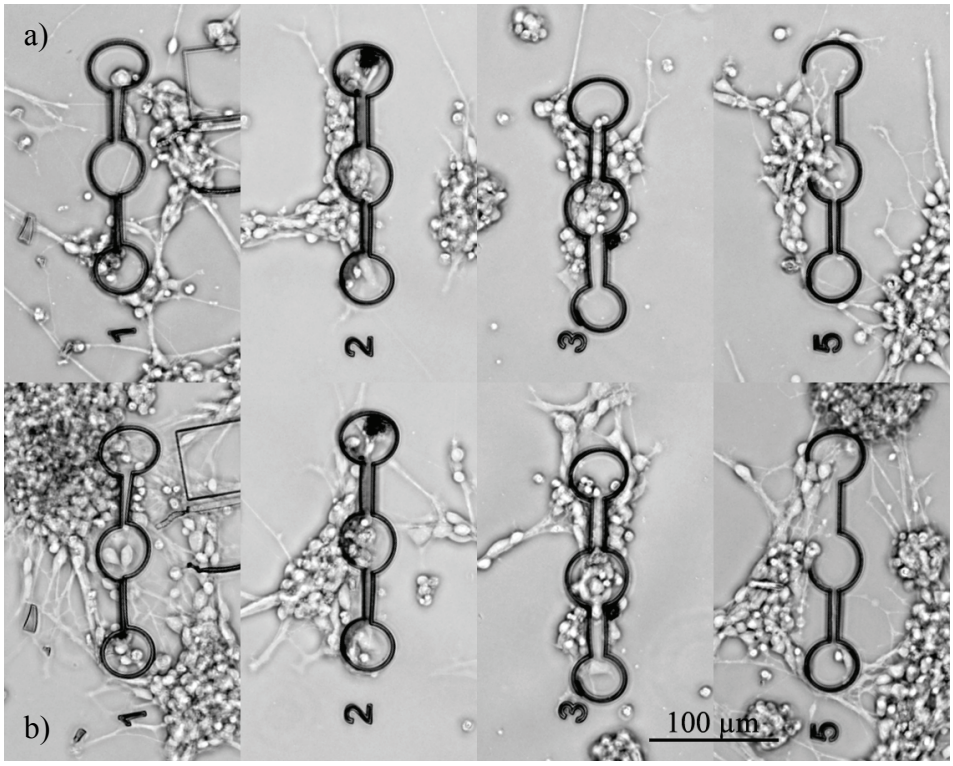



***Figure 39.*** *Individual neurocages on sample OA27 after a) two and b) three days in culture.*

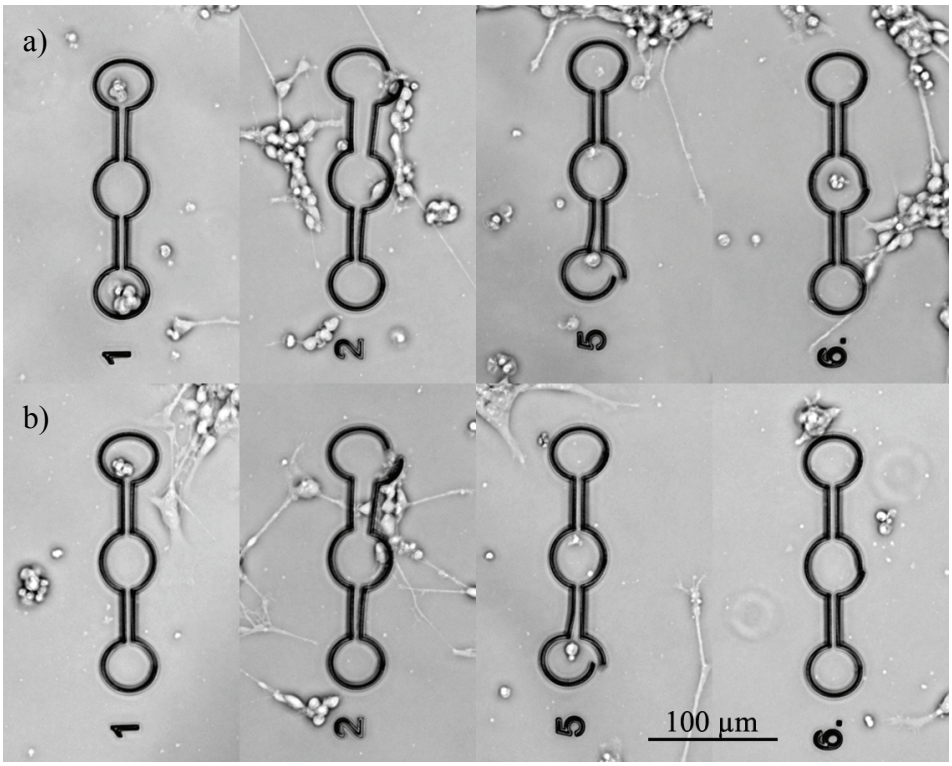



***Figure 40.*** *Individual neurocages on sample OA28 after a) two and b) three days in culture.*

The behaviour of the cells closely resembled that observed in experiment 2. It could be observed from Figures 37-40 that the cells still readily migrated towards the neurocages and even into them. Furthermore, when the cells attached to the surface inside the neurocages, they often extended their neurites along the tunnels of the neurocages. However, it was also evident that the neurocages did not guide the migration of the cells even though the laminin solution was carefully applied only into them. This result was further confirmed by immunocytochemically staining the samples. It was observed that live neurons were attached to the regions outside the neurocages, as shown in Figure 41.

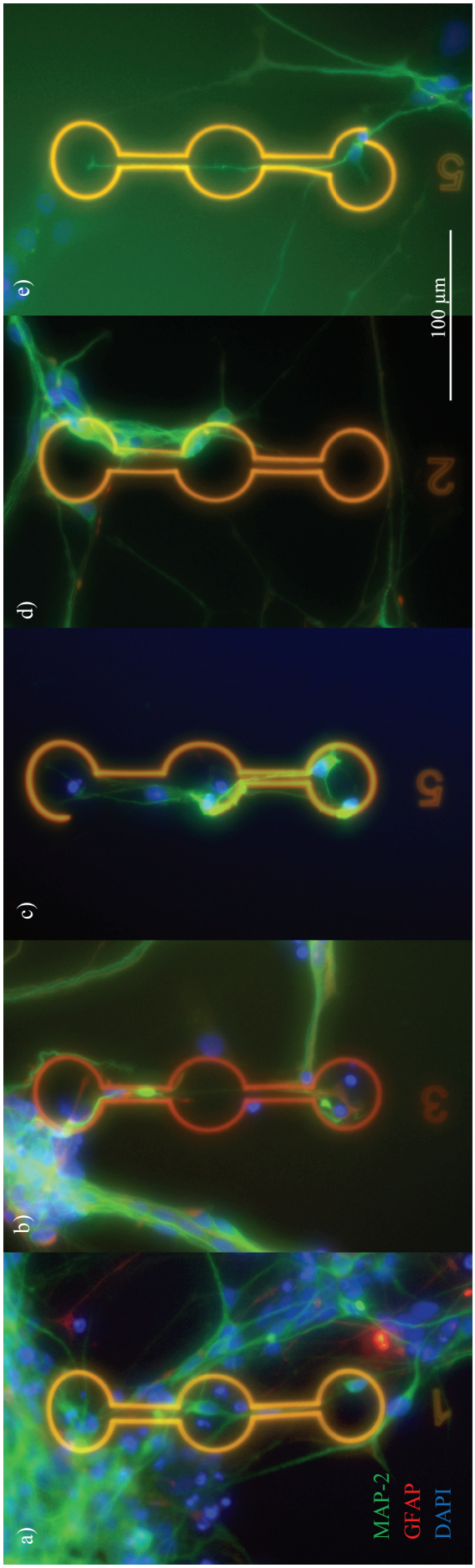



***Figure 41.*** *Immunocytochemically stained neurocages from samples a) OA25, b) OA26, c) OA27 and d-e) OA28 after five days in culture.*

Similarly to the previous experiments, the neurons seemed to be attracted to the neurocages. Specifically interesting was the observation that the cells migrated into the neurocages from the small gaps in the neurocage walls, as shown in the first two images from the right in Figure 41. This further emphasised the previous observation that although the cells preferred not to adhere to the surface inside intact neurocages, they readily migrated into them and then extended their neurites along the structures. Although the reason to this phenomenon was not known, it could be that the microenvironment inside the neurocages was somehow inhibiting the initial attachment of the cells. In fact, there had to be something severely inhibiting in the insides of the neurocages, as the cells attached better to the regions outside the neurocages even though these regions were not coated with the laminin solution. As this was the last experiment in the study, it was unfortunately not possible to further study the reasons and possible solutions to this problem.

# 6. DISCUSSION

Eight individual experiments were carried out during the study. The experiments were used to find the optimal methods to coat the samples with the laminin solution and to plate the cells that were to be cultured. Because the study included numerous experiments and the experiments were rather different from each other, the methods and results of them were summarised into Table 3 (see Appendices) to ease the discussion of the study.

## 6.1. Neurocages

The polymer-ceramic hybrid material Ormocomp® used in the neurocages had not been tested in a cell culture prior to this study. Ormocomp® was chosen as the material of the neurocages because it could easily be fabricated with two-photon polymerisation, it was biocompatible and it had previously been used in biomedical applications, such as microstructured medical devices [29]. Furthermore, although polymerised Ormocomp® was transparent, the neurocage structures could be visualised under a regular microscope in both air and solution. During this study it was found out that Ormocomp® was not harmful to neuronal cells differentiated from hESC. In fact, as the cells attached to the glass surface around the neurocages had a tendency to grow towards the neurocages, it was possible that Ormocomp® was an attractive substrate to the cells. Furthermore, the neurocages fabricated from Ormocomp® were solid and held their form, but they were also flexible enough to often return to their shape after distortions caused by the capillaries used in the application of the laminin solution. As an alternative material to Ormocomp® it is possible to use various hydrogels, as many of them are very compatible with cells. However, hydrogels are often quite soft materials and the lack of rigidity may become problematic when polymerising high walls. [48]

The dimensions of the neurocages were chosen with the neuronal cells and the possible MEA applications in mind. Additionally, similar dimensions had been previously utilised by Erickson and co-workers [11]. Although the nodes were quite large in comparison to the size of the cells and the dimensions have been successfully used before [11], it was possible that they were too small and hence inhibited the attachment of the cells onto the glass inside the neurocages. Furthermore, in previous studies the cells used have responded well to topographical guidance cues having the same dimensions as the neurocages [11, 13, 34, 39, 41] but in this study the neuronal cells migrated freely over the neurocage walls. A possible reason to this phenomenon is the use of human cells, which had not been used in the previous studies. To inhibit the

migration of the cells, it is possible to increase the wall height of the neurocages to at least 50 μm. However, the fabrication time of these neurocages is significantly higher when compared to the regular neurocages.

Two alternative neurocage designs were tested in this study. It was observed that the design A neurocages were very flexible, but retained their shape even after they were torn off the glass. The design B neurocages, in contrast, were very fragile. The walls of these neurocages easily collapsed or detached when the capillary tips came into contact with them. It was possible that the design A neurocages stayed more easily intact because the whole neurocage had a single continuous contour, whereas the design B neurocages were comprised of five separate contours.

During the study it was repeatedly observed that the cells initially inside the neurocages did not attach to the glass bottom. The reason for this phenomenon was not clear, but it was thought to be probable that the microenvironment inside the intact neurocages was different from the environment outside them. It might be useful to fabricate neurocages with some openings in the walls to increase the circulation of medium inside the intact structures and to possibly render the local microenvironment less inhibitory to the cells.

## 6.2. Application of the laminin solution

In the previous topographical guidance studies discussed earlier in this thesis (see Chapter 4.3.2 and Table 1 in Appendices) the structures were coated with an adhesive protein, but the coating method was generally not described in detail. This indicates that the structures were coated by some simple method, such as the droplet method used in this study. During this study three different application methods for the application of the laminin solution were tested. The first method tested was the droplet method, in which a droplet of the solution was placed on the sample, covering all the neurocages. The method was fast, simple and well reproducible. However, this method uniformly coated a relatively large region of the sample with laminin. This uniform coating was thought to affect to the free migration of the cells on the samples, although a similar phenomenon has not been reported in the previous studies with similar pattern dimensions [11, 13, 34, 39, 41]. As mentioned earlier, the human origin of the cells may also explain the different behaviour of the cells in this study.

In an attempt to apply the laminin solution more accurately into the neurocages, the simple droplet method was replaced by methods utilising micromanipulators. The micromanipulator set-up SU1 proved to be problematic because the Hamilton needle could not be fastened to the micromanipulator securely. Hence, the pressing of the piston of the needle caused the capillary tip to sway, which made the accuracy of the injection very poor. The swaying of the capillary tip also introduced a novel problem as it caused some of the neurocages to be torn off the glass. Furthermore, the piston of the Hamilton needle did not move very smoothly, which lead to the injection of droplets with very different volumes. If the accuracy problems could be overcome, SU1 would

be a good method to apply the laminin solution, though not very fast due to the careful application of pressure manually.

The micromanipulator set-up SU2 has been previously used in cell applications [26] and it was therefore tested also in this study. The set-up proved to be an excellent method for the application of the laminin solution. The navigation of the micromanipulator was very easy, even compared to the navigation of the micromanipulator in SU1. Furthermore, the application of the laminin solution could be performed with various injection pressure and injection time parameters. However, as various parameters could be used, there was very little information about the volume of the solution injected into the nodes. The solution never escaped from the neurocages with intact capillaries so apparently the volume of the neurocages was not reached. Although the injection volume probably varied from neurocage to another, the goal in the experiments utilising SU2 was only to cover the glass bottom inside the neurocages. Hence, there was no need to use a very specific injection volume.

The only minor setback of SU2 was the disappearance of the solution from some of the nodes after the application. However, it seems that this setback, too, was overcome in experiment 8, where the optimal temperature for the water bath was found. After the experiments it is evident that SU2 is the best method to apply the laminin solution if accurate application is wanted.

## 6.3. Application of the cell suspension

Various methods were used to fabricate the cell suspension used in the experiments. Trypsin generally produced a better single cell suspension than TrypLE, but trypsin is very harsh on the cells [14] and the filtered suspension therefore contained a large proportion of dead cells. Although TrypLE produced a suspension containing some cell aggregates, the aggregates could be removed by filtering the suspension before use. During experiment 8 it was observed that the cell suspension fabricated with TrypLE contained a large proportion of live single cells (see Figures 37-40). Therefore, the best cell suspension was achieved with TrypLE using two wells of neurospheres and a 50 µm filter.

A simple droplet method was tested to apply the cell suspension because the method was very fast and simple. However, the method was not successful, as the cells did not descend into the neurocages at all with this method. Therefore this method was not used to apply the cell suspension after the first experiment. Erickson and co-workers used a glass capillary (inner diameter 50 µm) attached to a manual syringe with their neurocage structures [11], which closely resembled the nodes of the neurocages in this study. The micromanipulator set-up SU1 was very similar to the equipment of Erickson and co-workers. However, the use of SU1 in the application of the cell suspension had the same problems as with the application of the laminin solution. The capillary tip swayed and the pressing of the piston caused variable volumes of the suspension to be injected. Additionally, the cell suspension blocked the capillary tip very easily, which

made the application method even more troublesome. It is not certain why the method was so unsuccessful in this study although it had been successful in the previous study [11]. However, it is apparent that the personnel applying the cells in the study were highly skilled and that the application process was highly optimised.

The injection of the cell suspension into the medium was a relatively successful method to apply the cell suspension, although the flow of the cells in the medium was rather random. With this method the largest amount of descended cells was often located on either side of the neurocage grid or even outside of the grid. The problem with the cell flow was overcome when a droplet of the cell suspension was injected into the tip of the needle and this droplet was gently set to the medium surface above the neurocages. This method was observed to be very fast and reliable, as the cells descended very accurate into and around the neurocages. Therefore, the droplet application method is very useful if accurate application of the cells only into the neurocages is not needed.

The set-up SU2 was not used to apply the cell suspension. However, the set-up has been successfully used to inject epithelial cell in previous studies [26]. Therefore, it might be useful to test SU2 with a filtered neuronal cell suspension in future studies. However, as the cells need to be injected into the nodes of the neurocages, the injection parameters need to be optimised to produce a fast pressure pulse in contrast to the steady injection used in the application of the laminin solution. It is also possible that the pressure pulse used to inject a cell into a single node will push out the cells already in that node. This possible drawback would make the use of SU2 in the application of the cells very difficult.

## 6.4. Cell culture

Neuronal cell cultures need an adequate amount of cells to be viable. In the study of Erickson and co-workers an additional population of neuronal cells was used to ensure the viability of the cells inside the neurocages. [11] Because the cell cultures used in the experiments of this thesis were relatively small, conditioned medium taken from another neuronal cell culture was tested in experiment 8. The conditioned medium had a definite positive effect on the cells, as most of the cells applied onto the samples attached to the glass surface. Furthermore, elaborate neuronal networks were visible on the samples after only two days in culture. If future studies are to be conducted, the use of conditioned medium is highly recommended.

Although the cells were live imaged before every medium change, the images did not give much information about the initial attachment and the migration of the cells. Additionally, the subsequent time points were days apart, so the imaging was not continuous. During the study it was not known for sure whether the cells initially inside the neurocages died or migrated out of the neurocages, although it was more likely that they died. Similarly, the migration of the cells into the neurocages was not recorded as the time points were so far apart. In future, it would be useful to continuously image the

cells for the first 12 hours after the application of the cell suspension to better understand the initial attachment and migration of the cells. A possible device for such an imaging is Cell-IQ (Chip-Man Technologies Ltd, Tampere, Finland).

# 7. CONCLUSIONS

The aim of this thesis was to study the effect of microstructures fabricated from a polymer-ceramic hybrid material on the growth, migration and neurite extension of neuronal cells differentiated from hESC. If the study was successful, such microstructures could in future be incorporated with MEA platforms to create small neuronal networks for the studies of neuronal network formation and function. The neurocage microstructures were fabricated by two-photon polymerisation onto microscope glass slide samples and coated with the ECM protein laminin. Subsequently, the cells were cultured atop the samples.

The study included eight individual experiments, which aimed to optimise both the methods for the application of the laminin solution and the cell suspension and the growth conditions of the cell population. Various application methods were tested and it was concluded that the micromanipulator set-up SU2 was the best method to apply the laminin solution. During this study a simple droplet application method proved to be the best method to apply the cell suspension. However, in future studies the possibilities of SU2 in the application of the cell suspension should be further studied.

During the experiments it was found out that the neuronal cells initially inside the neurocages did not attach to the glass bottom of the samples. In contrast, the cells outside the neurocages did attach to the glass and had a tendency to migrate towards the neurocages and even into them. Therefore, it was evident that the neurocages themselves or the material Ormocomp® was not harmful to the neuronal cells and it was speculated that the microenvironment inside the neurocages was somehow inhibitory to neuronal cell attachment. Furthermore, the cells that migrated into the neurocages readily extended their neurites along the tunnels and nodes of the structures. Some of the cells remained on the outsides of the neurocages, but extended their neurites into the neurocages. Such behaviour was observed especially around the neurocages that had gaps in their walls. Thus, it was concluded that the cells were attracted to the neurocages and readily followed the neurite extension guidance of the structures.

In future studies it should be first and foremost studied how the microenvironment inside the neurocages could be rendered less inhibitory to initial neuronal cell attachment. A possible solution is to fabricate a few openings into the neurocage walls to enable better circulation of the medium inside the neurocages. When the attachment of the cells into the neurocages is achieved, the tendency of the cells to migrate out of the neurocages also needs to be studied. If the cells migrate out of the neurocages as easily as they migrated into them in this study, it might be necessary to further optimise the dimensions of the neurocages.

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# APPENDICES

***Table 1.*** *A collection of studies utilising chemical and topographical neuronal guidance cues and three-dimensional cell-confining structures. w is the width, s the separation, d the depth, l the length and Ø the diameter of a guidance cue.*

| Guidance type | Material | Patterning technique | Dimensions of pattern | Cell population | Effect on cell adhesion | Effect on cell morphology | Ref. |
|---|---|---|---|---|---|---|---|
| Chemical patterns on substrate | Laminin or poly(D-lysine) (PDL) on glass; alternating lanes of PDL and Matrigel | Microfluidic patterning | - | Cortical neurons from E11-E14 mouse embryos | Adhesion confined to regions with PDL or laminin; more neurons on PDL than on Matrigel | Neurite growth was confined to regions with PDL or laminin, variation in reaction to the PDL/Matrigel border | [34] |

| Guidance type | Material | Patterning technique | Dimensions of pattern | Cell population | Effect on cell adhesion | Effect on cell morphology | Ref. |
|---|---|---|---|---|---|---|---|
| Chemical patterns on substrate | poly(L-lysine) (PLL) and fibronectin on poly(ethylene oxide) -like (PEO-like) surface | Microcontact printing | Pattern 1: square *w* = 100 µm, *s* = 100 µm; pattern 2: square *w* = 165 µm, *s* = 400 µm; single-cell culture square and line *w* = 10 µm, *s* = 10 µm | Neural stem cells from human umbilical cord blood (HUCB-NSC) | Cells specifically adhered to regions coated with PLL or fibronectin | Cells on PLL remained round and undifferentiated, cells on fibronectin flattened and extended neurites even in single-cell culture | [7] |
| | PLL or laminin/PLL on glass | Microcontact printing | Gradients comprised of protein rectangles: length from 0.9 µm, increments of 0.1 µm or 0.3 µm, *w* = 2-8 µm, *s* = 7.5 µm; a central node with *Ø* = 10 µm; gradient *l* = 100 µm or 200 µm | Cortical neurons from E18 rat embryos | Single neuron per gradient structure, located on the node | Gradient parameters significantly affected neurite growth, laminin/PLL and PLL produced mainly similar results | [15] |

| Guidance type | Material | Patterning technique | Dimensions of pattern | Cell population | Effect on cell adhesion | Effect on cell morphology | Ref. |
|---|---|---|---|---|---|---|---|
| Chemical patterns on substrate | PLL on MEA | Microcontact printing | Grid of lines: line $w$ = 2 µm, $s$ = 200 µm, nodes with Ø = 20 µm centred on line intersections | Hippocampal neurons from E18 rat embryos | 25 % of the cells plated attached onto nodes and lines | Formation of a functional neuronal network with higher cell plating densities | [24] |
| | Laminin and/or PLL on glass and MEA | Microdeposition / ink-jet deposition | Droplet Ø = 60-80 µm, on substrate mean spot Ø = 148 µm | Cortical neurons from E18 rat embryos | PLL alone enabled cell adhesion onto glass; both laminin and PLL were needed to enable adhesion onto MEA | Neurons formed connections with nearest neighbouring subpopulations (distance 50-150 µm) forming functional neuronal networks | [37] |

| Guidance type | Material | Patterning technique | Dimensions of pattern | Cell population | Effect on cell adhesion | Effect on cell morphology | Ref. |
|---|---|---|---|---|---|---|---|
| Topographical features on substrate | PDMS, coated with PDL | Micromoulding | Groove $d$ = 2.5-69 µm, $w$ = 50-350 µm | Cortical neurons from E11-E14 mouse embryos | - | Groove depth affected cellular response, width had no effect | [34] |
| | Photosensitive poly(imide) (PSPI) on glass, coated with collagen | Photolithography | Groove $d$ = 11 µm, $w$ = 20-60 µm, $l$ = 400 µm, $s$ = 10 µm | PC12 cells, source not mentioned | - | Groove width affected neurite orientation, neurite alignment after contact to wall, cells inside grooves had less neurites than controls | [39] |

| Guidance type | Material | Patterning technique | Dimensions of pattern | Cell population | Effect on cell adhesion | Effect on cell morphology | Ref. |
|---|---|---|---|---|---|---|---|
| Topographical features on substrate | Poly(L-lactide) (PLLA), coated with laminin | Polymer casting | 2x2 $cm^2$ samples, pattern cross-section sinusoidal; sample 1: pattern repetition 1 µm, groove mean $d$ = 150 nm; sample 2: pattern repetition 2 µm, groove mean $d$ = 215 nm | Rat PC12 cells, sympathetic ganglia neurons from E7-E8 chicken embryos | - | Neurites of both cell types aligned parallel to the grooves, sympathetic ganglia neurons elongated longer neurites on grooved substrate, neurite number was not affected | [33] |
| | Poly(lactide-*co*-glycolide) (PLGA), coated with collagen I or laminin peptide | Laser ablation | Sample 1: groove $w$ = 5 µm, $d$ = 2 µm, $s$ = 5 µm; sample 2: groove $w$ = 10 µm, $d$ = 2-3 µm, $s$ = 10 µm | PC12 cells | - | Both grooves guided neurite orientation, more parallel neurites in thinner grooves, cells inside grooves had less neurites than controls | [51] |

| Guidance type | Material | Patterning technique | Dimensions of pattern | Cell population | Effect on cell adhesion | Effect on cell morphology | Ref. |
|---|---|---|---|---|---|---|---|
| Topographical features on substrate | Porous and dense PLLA, coated with PLL | Polymer casting | Two patterns, mould dimensions: pattern 1: groove $w = 30$ µm, $d = 20$ µm, $s = 20$ µm; pattern 2: groove $w = 20$ µm, $d = 15$ µm, $s = 10$ µm, 10 µm intersections every 100 µm | Hippocampal neurons from P1-P3 hamsters | - | Neurons were localised in grooves, neurites grew aligned with the grooves, membrane porosity and/or dimensions affected neurite growth and orientation | [41] |
| Structures confining cells | SU-8 on silicon oxide, coated with laminin | Photolithography | Wall $h = 15$ µm; square chamber size 40x40-120x120 µm; corridor $l = 700$ µm, $w = 20$-50 µm | DRG neurons from E10 chicken embryos | - | Chambers, but not corridors, inhibited neuritogenesis of neurons; axons were able to escape from square chambers; axons turned around obtuse rather than sharp-angled corners | [13] |

| Guidance type | Material | Patterning technique | Dimensions of pattern | Cell population | Effect on cell adhesion | Effect on cell morphology | Ref. |
|---|---|---|---|---|---|---|---|
| Structures confining cells | SU-8 photoresist on MEA, coated with laminin and PDL | Photolithography | Cluster structure $h$ = 350 µm, chamber $Ø$ = 3 mm, connecting channel $w$ = 300 µm, $l$ = 800 µm | Cortical neurons from E18 rat embryos | - | Synchronous activity occurred only inside the clusters; a stimulus propagated from one cluster to another | [4] |
| | Parylene on MEA coated with poly(ethylene)-imine and laminin | Photolithography | Neurocage $Ø$ = 40 µm, $h$ = 9 µm, $s$ = 110 µm; tunnel $w$ = 10 µm, $h$ = 1 µm, $l$ = 25 µm | Hippocampal CA1 and CA3 pyramidal cells from E18 rat embryos | 11 out of 16 neurons in neurocages adhered and grew | Somata stayed inside the neurocages, neurites grew out from the neurocages and formed a network | [11] |

***Table 3.*** *Summary of the experiments carried out in this study.*

| Experiment | Sample | Laminin solution and the application method | Laminin application results | Cell suspension and the application method | Cell suspension application results | Cell culture results |
|---|---|---|---|---|---|---|
| Experiment 1 | OA1 | 20 µg/ml, 20 µl droplet | Bubbles inside some nodes, disappeared during incubation | Cell suspension from one well of neurospheres with TrypLE, injected into medium | Only a few cells inside the nodes | No living cells after five days in culture |
| | OA2 | | | Cell suspension from one well of neurospheres with TrypLE, droplets injected onto the neurocages | Very few cells inside the nodes | |
| | OA3 | | No bubbles inside the nodes | Cell suspension from two well of neurospheres with TrypLE, injected into medium | Quite promising, a good amount of cells inside the neurocages | Some attached cells inside the structures after three days, all cells dead after seven days |
| | OA4 | | | | | |
| | OA5 | | | | | |

| Experiment | Sample | Laminin solution and the application method | Laminin application results | Cell suspension and the application method | Cell suspension application results | Cell culture results |
|---|---|---|---|---|---|---|
| Experiment 2 | OA6 | 20 µg/ml, 20 µl droplet | No bubbles inside the nodes | Cell suspension from four wells of neurospheres with TrypLE, injected into medium | A varying amount of cells inside the neurocages, no distinctive factor causing the differences | Cells grew freely in and out of the neurocages, a tendency to grow towards them; the cells inside the cages extended neurites along the tunnels |
| | OA7 | | | | | |
| | OA8 | | | | | |
| | OA9 | | | | | |
| | OA10 | | | | | |
| | OA11 | | | | | |
| | OA12 | | | | | |
| | OA13 | | | | | |
| | OA14 | | | | | |
| | OA15 | | | | | |

| Experiment | Sample | Laminin solution and the application method | Laminin application results | Cell suspension and the application method | Cell suspension application results | Cell culture results |
|---|---|---|---|---|---|---|
| Experiment 3 | OA16 | 20 µg/ml, applied with SU1 | Inaccurate injection of the solution, the solution evaporated in seconds | - | - | - |
| | OA17 | 20 µg/ml, 20 µl droplet | No bubbles inside the nodes | Cell suspension from two wells of neurospheres with TrypLE, injected into a droplet of medium | Cells burst out of the capillary, descended randomly | - |
| | OA18 | | | Cell suspension from two wells of neurospheres with TrypLE, applied with SU1 | Inaccurate injection of the suspension, evaporation in seconds | - |
| | OA19 | 20 µg/ml, 20 µl droplet | No bubbles inside the nodes | Cell suspension from two wells of neurospheres with trypsin, applied with SU1 | Suspension blocked the capillaries | - |

| Experiment | Sample | Laminin solution and the application method | Laminin application results | Cell suspension and the application method | Cell suspension application results | Cell culture results |
|---|---|---|---|---|---|---|
| Experiment 4 | OA20 in a water bath | 20 µg/ml, 20 µl droplet | No bubbles inside the nodes | Cell suspension from two wells of neurospheres with trypsin, applied with SU1 | Cells randomly dispersed, some inside the neurocages | Cells inside the neurocages did not attach to the glass, no neuronal cells even on the regions outside the neurocages |
| | OA21 in a water bath | 100 µg/ml, 20 µl droplet | | Cell suspension from two wells of neurospheres with trypsin, 10 µl droplet | | |
| Experiment 5 | OA22 | Laminin in the cell suspension | - | Cell suspension from two wells of neurospheres with trypsin, addition of laminin to a concentration of 20 µg/ml, filtered with a 50 µm filter, applied with SU1 | Inaccurate injection of the suspension, the capillary tip tore some neurocages off the glass | Lots of spherical cells floating in the medium, no attachment of the cells onto the glass inside or outside the neurocages |
| | OA23 in a water bath | | | | | |

| Experiment | Sample | Laminin solution and the application method | Laminin application results | Cell suspension and the application method | Cell suspension application results | Cell culture results |
|---|---|---|---|---|---|---|
| Experiment 6 | OA24 in a water bath, not sterile | 20 µg/ml, applied with SU2 | Very accurate application of the solution into the nodes | Cell suspension from two wells of neurospheres with trypsin, filtered with a 50 µm filter, injected onto the surface of the medium | Cells accurately atop the neurocages, descended well into the neurocages | Live neuronal cells on the sample, not inside the neurocages |
| Experiment 8 | OA25 in a water bath | 20 µg/ml, applied with SU2 | Very accurate application of the solution | Cell suspension from two wells of neurospheres with TrypLE, filtered with a 50 µm filter, injected onto the surface of the medium | Cells accurately atop the neurocages, descended well into the neurocages | Cells outside the neurocages formed elaborate networks, the cells migrated towards and into the neurocages, cells inside the neurocages extended neurites along the tunnels |
| | OA26 in a water bath | | Rapid disappearance of the solution from the nodes, had to be added several times | | | |
| | OA27 in a water bath | | | | | |
| | OA28 in a water bath | | Very accurate application of the solution | | | |

| Experiment | Sample | Laminin solution and the application method | Laminin application results | Cell suspension and the application method | Cell suspension application results | Cell culture results |
|---|---|---|---|---|---|---|
| Experiment 7 | OB2 in a water bath | 50 µg/ml, applied with SU2 | Rapid disappearance of the solution from the nodes, had to be added several times, OB3 completely covered with the solution | Cell suspension from two wells of neurospheres with trypsin, filtered with a 50 µm filter, injected onto the surface of the medium | Cells accurately atop the neurocages, descended well into the neurocages | Cells in the neurocages did not attach, large aggregates of cells atop the neurocages |
| | OB3 in a water bath | | | | | Sample was covered with cells, lots of neuronal cells, the cells inside the neurocages did not attach |
| | OB4 in a water bath | | | | Neurocages torn off before the application of the cell suspension | - |
| | OB5 in a water bath | | | | Cells accurately atop the neurocages, descended well into the neurocages | Cells in the neurocages did not attach, large aggregates of cells atop the neurocages |